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Role of TcyB and Gpo in the maintenance of redox homeostasis and adaptation to oxidative stress in *Lactobacillus sanfranciscensis*

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ABBREVIATIONS

Ahp	alkyl hydroperoxide reductase gene
approx.	approximately
BCAA	branched chain amino acid
bp	base pair
C.	*Candida*
CDM	chemical defined media
cfu	colony forming unit
Cye3	cyanine 3
Cye5	cyanine 5
DSM	Deutsche Sammlung von Mikroorganismen
DTT	dithiothreitol
E.	*Escherichia*
EDTA	ethylene diamine tetraacetic acid
FPKM	Fragments Per Kilobase of transcript per Million mapped reads
g (centrifugation)	relative centrifugal force
g (weight)	gram
Gpx, Gpo	glutathione peroxidase gene
GSH	glutathion (reduced)
GshR	glutathion reductase
GSSG	glutathion (oxidized)
h	hour
H_2O_2	hydrogen peroxide
HPLC	high-performance liquid chromatography
L.	genus *Lactobacillus*
LAB	Lactic acid bacteria
LB	lysogeny broth
lox	lactate oxidase gene
M	molar
min	minute
Mn^{2+}	manganese

mMRS	modified deMan, Rogosa, Sharp medium
NCBI	national center for biotechnology information
nox	NADH oxidase gene
npx, *npr*	NADH peroxidase gene
$O_2^{\bullet-}$	superoxide radical
OD	optical density
ORP	Oxidation- reduction potential
p	probability
PCR	polymerase chain reaction
perR	peroxide- responsive repressor gene
PIPES	piperazine-N,N′-bis(2-ethanesulfonic acid)
pox	pyruvate oxidase gene
(p)ppGpp	guanosine- 3'- diphosphate- 5'- triphosphate
rex	redox- sensing transcriptional repressor
ROS	reactive oxygen species
rpm	revolutions per minute
RT	room temperature
s	second
SOD, *sod*	superoxide dismutase gene
spp.	*Species*
spx	transcriptional regulator gene
tcyB	cystine transport permease gene
TMW	Technische Mikrobiologie Weihenstephan
trxA	thioredoxin
trxR, *trxB*	thioredoxin reductase
V	volt
vs.	versus
w/ v	weight/ volume
WT	wildtype

CONTENTS

1 INTRODUCTION

1.1 General information about lactic acid bacteria

Lactic acid bacteria (LAB) have been used in food production (sourdough, vegetable fermentations, cheese, meat, beer, wine etc.) since prehistoric times. The genus *Lactobacillus* comprises of gram-positive, non- spore forming, catalase negative (pseudocatalase rarely found) and acid tolerant bacteria with a rod or cocci shape and less than 55 mol% GC content (Stiles & Holzapfel 1997; Kandler 1983). LAB require complex nutritional sources for optimal growth. Due to their incomplete electron transport chain (ETC) and citric acid cycle, fermentation with generation of ATP via substrate level phosphorylation resembles the favored way for energy production.

Depending on their resulting carbohydrate fermentation spectra, LAB have been divided into three groups (Hammes & Vogel 1995):

- Group I: obligately homofermentative LAB
- Group II: facultatively heterofermentative LAB and
- Group III: obligately heterofermentative LAB

LAB of the first group use the Embden- Meyerhof (EM) pathway to oxidize imported hexoses to pyruvate with formation of ATP. Gluconate and pentoses are not metabolized. Facultatively heterofermentative LAB resemble the second group as most of the hexoses are converted into lactic acid. They are able to utilize pentoses because they can induce the enzyme phosphoketolase which yields acetic acid. LAB of the third group ferment hexoses to lactic acid, CO_2 and ethanol, whereas pentoses are metabolized to lactic and acetic acid. This group uses the phosphoketolase pathway for fermentation of hexoses and pentoses in general for energy production. If additional electron acceptors (oxygen, citrate, malate, tartrate, fructose etc.) are present, acetate formation can yield an additional ATP. Acetate is formed by decarboxylation of pyruvate to acetyl phosphate by pyruvate oxidase (*pox*). In *L. sanfranciscensis*, acetyl phosphate is dephosphorylated to acetate by acetate kinase reaction (*ack*). Thus, the presence or absence of external available electron acceptors decides, if formation of ethanol or acetate is favored (Knorr et al. 2001).

Generally, the oxidation of hexoses requires available redox carriers (NAD^+/ NADH) which are reduced by electron acquisition ($NAD^+ \rightarrow$ NADH). The effective fermentation of imported sugars requires sufficient NADH which is re- oxidized to NAD^+ (e.g. in lactate dehydrogenase reaction which forms lactic acid from pyruvate). In LAB, the entry of oxygen yields additional ATP, whereas under anaerobic conditions formation of ethanol results in reoxidized NAD^+. One explanation is that during aerobiosis the enzyme NADH oxidase (*nox*) competes with acetaldehyde dehydrogenase and alcohol dehydrogenase for NADH. Consequently, the strain decides depending on growth stage and environmental stimuli if ATP formation or regeneration of coenzymes is preferred.

LAB are found in nutritious environments like milk/ milk products, on plants, fruits, skin, mucosal tissue and in the gastrointestinal tract (GIT) of humans, animals and insects. Therefore, they are exposed to different environmental stressors like oxygen, low pH, high osmotic pressure, temperature, competitive (pathogenic) organisms etc.. Depending on the ecological origin, different strains of LAB acquired different mechanisms to combat different stimuli. The response to these effectors is therefore determined on strain level.

1.2 Redox reactions and oxidation- reduction potential (ORP)

Redox reactions comprise of all reactions where the transfer of electrons from electron donor to electron acceptor occurs. During this coupled reduction/ oxidation reaction, the electron donor becomes oxidized (release of electrons, X) and the electron acceptor is reduced (uptake of electrons, Y) as follows:

$$X_{red} + Y_{ox} \leftrightarrow X_{ox} + Y_{red}$$

Thus, oxidation- reduction enzymes are involved in numerous reactions in bacteria. These include enzymes, which reduce oxidized substrates, dehydrogenases, oxidoreductases, enzymes with a role in ETC and others.

The characteristic of compounds release or uptake of electrons (power of electron affinity of a redox pair) can be quantitatively described as oxidation– reduction or oxidoreduction potential (ORP) potential (= redox/ reduction potential E_h). The real- time measurement during fermentations can be carried out with ORP electrodes. During a spontaneous redox reaction, the redox pair with the lower ORP functions as electron donor whereas the redox pair with a higher ORP serves as electron acceptor. Each redox pair exhibits its own ORP value. The difference in ORP between electron donor and acceptor is often expressed as ΔE_h. The simultaneous presence of a suitable electron donor and electron acceptor is the prerequisite for energy production and release. An example for intracellular redox pairs are NADH/ NAD^+ and NADPH/$NADP^+$ whereas the ORP is mainly determined by the ratio of NADH/ NAD^+ (Liu et al. 2012).

In literature, different assumptions exist about the reasons for a decrease in E_h values in bacterial fermentations. The presence of external sulfhydryl groups (Oktyabrsky & Smirnova 1993; Michelon et al. 2010) is proposed besides the activity of bacterial enzymes involved in ETC including NADH oxidase (Tachon et al. 2010).

1.3 General aspects about the oxidative stress response in LAB

1.3.1 Sources of reactive oxygen species and detoxification mechanisms

Depending on their natural habitat, LAB are exposed to low or high concentrations of oxygen. The resistance is based on the presence or absence of detoxifying enzymes against emerging reactive oxygen species (ROS) and varies between different LAB. A general overview of the possible formation of predominant ROS during aerobiosis in LAB is illustrated in Figure 1 and has been reviewed recently (Pedersen et al. 2012).

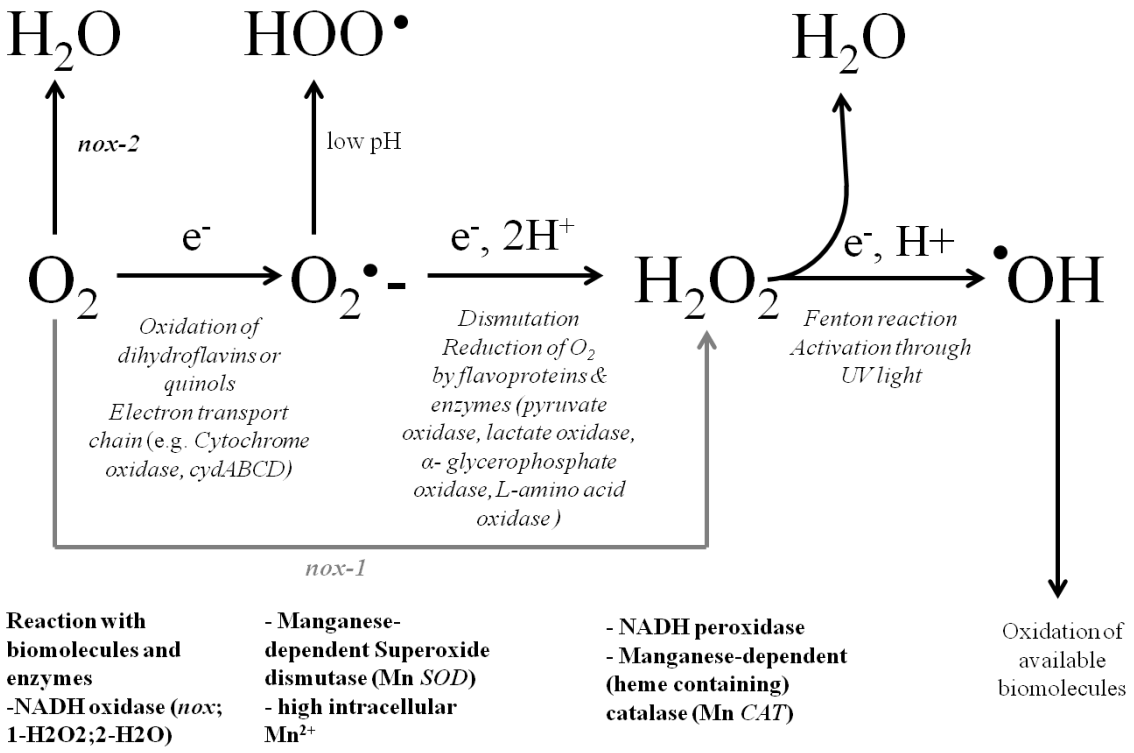


Figure 1: Schematic representation of the predominant ROS formation pathways during aerobic metabolism in LAB. Visible in italics are the biomolecules which participate in ROS formation, at the bottom and marked in bold are the enzymes/ compounds which are used for elimination of the corresponding ROS.

Due to incomplete biosynthetic pathways, most LAB are unable to synthesize heme or cytochromes, which are necessary for energy- linked oxygen metabolism. It is described for *L. plantarum*, *L. rhamnosus*, *L. brevis*, *L. paralimentarius* and others that aerobic growth can be stimulated by addition of heme and menaquinone (Brooijmans et al. 2009). Despite the fact that aerobic metabolism produces ROS, which can lead to growth stagnation, several LAB respond with enhanced survival, increase in growth yield, elevated biomass production and elevated resistance to hydrogen peroxide (H_2O_2) (Watanabe et al. 2012; Archibald & Fridovich 1981; Duwat et al. 2001; Gaudu et al. 2002).

Oxygen itself can easily react with different enzymes either involved in ETC like cytochrome oxidases or others like e.g. or flavinoxidases (NADH oxidase) to superoxide radicals ($O_2^{\bullet-}$) or H_2O_2 directly.

Hydride ions from organic substrates reduce the flavins which transfers electrons to specific locations (iron- sulfur clusters or quinones) within the enzyme. Oxygen can react at this step with the reduced flavins which results in formation of ROS (ImLay 2003).

The generated superoxide radicals can be detoxified in LAB by superoxide dismutase (mainly manganese- containing SOD, *sodA*) with generation of H_2O_2. LAB which lack SOD evolved the mechanism to accumulate high concentrations of intracellular manganese (Mn^{2+}) which acts as a scavenger for ROS in general (Archibald 1986). Thus, the divalent cation fulfils essential antioxidative functions as cofactor in SOD (Archibald & Fridovich 1981), catalase (Kono & Fridovich 1983) and as "free" intracellular Mn^{2+} (Archibald & Duong 1984). Corresponding reactions for SOD and Mn^{2+} are:

$$Sod \text{ and/ or } Mn^{2+}: 2\ O_2^{\bullet-} + 2\ H^+ \rightarrow H_2O_2 + O_2$$

H_2O_2 can also be produced by various oxidases like NADH oxidase (*nox*), lactate oxidase (*lox*) and pyruvate oxidase (*pox*) (De Angelis & Gobbetti 2004). NADH oxidases have already been characterized in several lactobacilli, either producing H_2O_2 or H_2O (Y.- W. Zhang et al. 2012; Torre & Garel 2000b; Jänsch et al. 2011; Geueke & Riebel 2002) as indicated in Figure 1 and in the following formula:

$$nox\text{- 1}: NADH + H^+ + O_2 \rightarrow NAD^+ + H_2O_2$$

$$nox\text{- 2}: 2\ NADH + 2\ H^+ + O_2 \rightarrow 2\ NAD^+ + 2\ H_2O$$

Lactate and pyruvate oxidases are not widely spread in LAB but known for their H_2O_2 producing ability especially under aerobic conditions (Zitzelsberger 1984; Taniai et al. 2008; Seki et al. 2004a; Stevens et al. 2010). Corresponding reactions include:

$$lox: \text{lactate} + O_2 \rightarrow \text{pyruvate} + H_2O_2$$

$$pox\ (spxB): \text{pyruvate} + P_i + O_2 \rightarrow \text{acetyl-P} + CO_2 + H_2O_2$$

Pyruvate can further be converted into acetate and ATP from acetyl-P, which is catalysed by acetate kinase (*ack*) reaction already known for LAB (Knorr, Ehrmann 2001):

$$ack: \text{acetyl-P} + ADP \rightarrow \text{acetate} + ATP$$

As the formation of H_2O_2 can lead to cessation of growth, the presence of efficient detoxification mechanisms influences the survival ability of the strain. As LAB lack the enzyme catalase which especially eliminates high concentrations of H_2O_2, enzymes like NADH peroxidase (*npr*, *npx*) and alkyl hydroperoxide reductases (*ahp*) are found, which catalyze the following reactions:

$$npr, npx: NADH + H^+ + H_2O_2 \rightarrow 2\ H_2O$$

ahp: organic hydroperoxide (or H_2O_2) + NADH + H^+ → (alcohol) + NAD^+ + H_2O

In general, peroxidases require electron donors, the elimination of H_2O_2 therefore always depends on the NADH pool which is provided from metabolism alluded before (Imlay 2013). In *L. panis* PM1 most of the produced H_2O_2 was formed via *nox*, which was expressed under aerobic and microaerobic conditions whereas *npx* was activated in the presence of oxygen (Kang et al. 2013). The authors propose that rather reoxidation of NADH in a coupled *nox*/ *npx* reaction is responsible for the resistance mechanisms than ethanol production as already known from other heterofermentative LAB. *Ahp* can detoxify H_2O_2 and organic hydroperoxides and have been described in *Enterococcus faecalis* (La Carbona et al. 2007), *Streptococcus mutans* (Poole et al. 2000), *Bacillus subtilis* (Bsat et al. 1996) and others.

Besides activity of different *ahp* or *npx*, a manganese dependent (pseudo) catalase exists in a strain of *L. plantarum* which prevented accumulation of H_2O_2 (Kono & Fridovich 1983; Condon 1987).

1.3.2 Enzymes involved in thiol metabolism contribute to oxygen tolerance

The prevention of ROS through enzymatic and non- enzymatic defense mechanisms is one way to minimize intracellular damages in the organisms in general. Enzymes involved in the thiol metabolism like glutathione- glutathione peroxidase system (Jänsch et al. 2007), the thioredoxin- thioredoxin reductase system (Van De Guchte et al. 2002; Vido, Diemer, Dorsselaer, et al. 2005; Rocha et al. 2007) as well as cyst(e)ine uptake and metabolism (Turner et al. 1999; Lo et al. 2009; Hung et al. 2005) are known for their "antioxidative" actions in LAB.

1.3.2.1 Glutathione- glutathione peroxidase (gpo, gpx)

The general role of glutathione (GSH) in LAB has been reviewed recently (Pophaly et al. 2012a) and their glutathione accumulation potential is already known (Wiederholt & Steele 1994; Kullisaar et al. 2002).

The enzyme glutathione peroxidase (*gpo*, *gpx*) eliminates organic hydroperoxides (ROOH) and/ or H_2O_2 with formation of water coupled with the oxidation of GSH to oxidized glutathione (GSSG) whereas the GSH/ GSSG redox switch determines the oxidative status of the bacterial cell (Jones 2002).

gpo, *gpx*: 2 GSH + H_2O_2 → GSSG + $2H_2O$

and/ or

ROOH + 2GSH → ROH + GSSG + H_2O

Gene deletion studies regarding *gpx* in gram- positive bacteria are limiting. A *gpxA* insertion mutant in *Neisseria meningitides* showed increased sensitivity to oxidative stress caused by superoxide generating compound paraquat and slightly increased sensitivity after H_2O_2 treatment, whereas aerobic growth was unaffected (Moore 1996). In *Streptococcus pyogenes*, a *gpoA* mutant showed no increased sensitivity to oxygen but to paraquat (King et al. 2000) and seems to be essential for pathogenicity in murine models mimicking suppurative diseases (Brenot et al. 2004).

So far no deletion studies exist of a *gpo* (*gpx*) mutant in *Lactobacilli*, however the role of glutathione reductase (*gshR*), which is necessary for reduction of GSSG, was already investigated. A glutathione reductase (Δ*gshR*) mutant of *L. sanfranciscensis* DSM20451^T showed growth defects in the presence of oxygen and paraquat in MRS media without added cysteine (Jänsch et al. 2007). It could be observed that a high intracellular GSH/ GSSG status in which *gshR* is essential, improves the strains ability to defend against emerging oxidative stress. A deletion of *gshR* lead to decreased thiol levels in sourdough, therefore *gshR* in *L. sanfranciscensis* seems to be essential for the increase in thiol groups which is supported by the fact, that this enzyme is expressed during sourdough fermentation (Jänsch et al. 2007).

1.3.2.2 Thioredoxin reductase (trxB, trxR) and glutaredoxin-like protein (nrdH)

The bacterial thioredoxin system has been intensively reviewed (Zeller & Klug 2006; Lu & Holmgren 2013). The thioredoxin reductase (*trxB*, *trxR*) as oxidoreductase has essential functions in the reduction of small proteins, called thioredoxins (*trxA*), which act as defense proteins during oxidative insults for maintenance of a reducing intracellular milieu. The reaction back into the reduced forms lead to oxidation of active cysteine residues which form the redox- active center of the protein.

The typical thioredoxin-fold, which can also be found in several proteins involved in redox reactions, consists of a four- stranded central β-sheet which is flanked by three α-helices (Martin 1995). A structural similarity within this thioredoxin-fold is the CXXC motif where two cysteines (C) embed two variable amino acids (X). The role of the cysteines during oxidation is described in chapter 1.6.

The importance of the function of thioredoxins and thioredoxin reductases becomes apparent evaluating the effects in deletion mutants. In many cases gene inactivation fails because of essentiality of these proteins for growth and survival in a wide range of bacteria, especially those which lack other "antioxidative" enzymes. This is supported by the fact that successful gene deletions of thioredoxin reductase in particular are described quiet rarely or growth is highly dependent on the presence of reducing substances like cysteine, GSH or dithiothreitol (DTT) (Rocha et al. 2007; Vido, Diemer, Van Dorsselaer et al., 2005).

As a *trxB* deletion in *Staphylococcus aureus* causes lethality, the effect of *sarA* deletion, which normally controls transcription of many virulence- associated genes (*trxB* amongst others), was evaluated (Ballal & Manna, 2010). Transcription of *trxB* was enhanced under aerobic and

microaerophilic conditions and with an effect after diamide challenge comparing the *sarA* mutant with the wildtype.

Similar observations could be seen for a *trxB* mutant of *L. casei* strain Shirota. Growth under aerobic conditions was diminished and sensitivity against H_2O_2 and disulfide stress was increased (Serata et al. 2012). A *trxB* mutant of *Bacteroides fragilis* was unable to grow in media without addition of cysteine or dithiothreitol (DTT). Further, sensitivity against diamide and oxygen was increased (Rocha et al. 2007). A *trxB1* inactivation in *Lactococcus lactis* resulted in accumulation of H_2O_2, whereas growth defects could be partly restored by addition of GSH, cysteine and pyruvate (Vido, Diemer, Van Dorsselaer et al. 2005). In contrast to these findings, overexpression of *trxB1* in *L. plantarum* WCFS1 improved the tolerance against oxidative stress originating from H_2O_2 and diamide (Serrano et al. 2007 a). Further, expression of genes involved in synthesis of purine and sulfur- containing amino acids, energy metabolism, stress response and Mn^{2+} transport were upregulated with *trxB1* overexpression and H_2O_2 treatment (Serrano et al. 2007a).

Mutations in the thioredoxins can also have distinct effects. Diminished growth after application of oxygen, H_2O_2, paraquat and nitrosative stresses could be observed in a dysfunctional *trxA1* gene of *Helicobacter pylori*. A *trxA* inactivation in *Bacillus subtilis* resulted in cysteine/ methionine auxotrophy, defects in endospore formation and cytochrome C synthesis (Möller & Hederstedt 2008). Consequently, the role of *trxA* as electron donor for different cellular processes and its relevance in sulfate assimilation is clearly evidenced.

Glutaredoxins are thiol- disulfide oxidoreductases, which belong to the thioredoxin superfamily with the typical CXXC motif which becomes reversibly oxidized. These small proteins share homologies with *nrdH* proteins which function as hydrogen donor for the *nrdEF* ribonucleotide reductase. *NrdH* proteins could especially be found in several bacteria which lack GSH (Stehr & Lindqvist 2004; Jordan, Pontis, Fredrik, Hellman, Gibert, 1996). They are often named as glutaredoxin-like proteins, primarily due to the lack of suitable amino acids, which are responsible for GSH binding (Bushweller et al. 1994) and secondly due to the absence of GSH for reduction of disulfide bonds between the cysteines as already known for *Lactococcus lactis* (Jordan, Pontis, Fredrik, Hellman, Gibert, 1996). In this strain, the coding operon contains the *nrdEF* genes and two open reading frames, of which one is *nrdH*. In *Escherichia (E.) coli* the oxidized *nrdH* proteins become, similar to thioredoxins, reduced back via *trxR* whereas NADPH serves as electron donor (Jordan A, Aslund F, Pontis E, Reichard P, Holmgren 1997).

1.3.2.3 Cystine transport in gram- positive bacteria

Bacterial cystine transport systems exhibit a high specificity for cystine. The role of cystine transporters have already been described in *Bacillus subtilis* (Burguière et al. 2004), *L. reuteri* BR11 (Hung et al. 2005; Lo et al. 2009) and *E. coli* (Berger & Heppel 1972). Cysteine helps in protein

folding by forming disulfide bonds; it acts in catalytic sites of enzymes, and has diverse functions as a precursor of many molecules (methionine, GSH, biotin, coenzyme A, thiamine etc.).
Previous work in other gram- positive bacteria indicates the participation of the cysteine/ cystine transport in oxidative stress response. Severe growth defects in media without cysteine during aerobiosis and in the presence of paraquat could be observed for a *L. sanfranciscensis* DSM20451^{T}Δ*gshR* and a *L. sanfranciscensis* DSM20451^{T}Δ*nox* mutant which were also sensitive to diamide treatment (Jänsch et al. 2007; Jänsch et al. 2011). The effects could be restored when cysteine was added to the media due to the fact that *L. sanfranciscensis* imports cyst(e)ine to increase thiol levels (Jänsch et al. 2007). However, it is unclear to which extent cystine transport is required as nutritional source, contributes to intracellular thiol homeostasis and/ or is involved in actions against oxidative stress.

Besides intracellular cysteine and cystine transport, especially *Firmicutes* tend to exclude cysteines from exported proteins, which can exhibit a selective advantage in extreme redox environments for example in presence of oxidative substances (Daniels et al. 2010). It is confirmed with the finding that an increase of exofacial thiol groups which are located on exoproteins in *Lactococcus lactis* leads to a decrease of the redox potential (Michelon et al. 2010).

Effects of gene inactivation of cyst(e)ine transporters in gram negative bacteria overlap with activities in gram- positive bacteria and shows also the rate of interchangeability of cysteine and cystine. *E. coli* mutants defective in cysteine transport (Δ*ydeD*) or cystine binding protein (Δ*fliY*) showed increased sensitivity to H_2O_2 challenge compared to the wildtype (Ohtsu et al. 2010). The growth of Δ*fliY* was completely abolished using 0.5 mM H_2O_2**.** In agreement with these facts, the deletion of the cystine uptake gene (*cyuC*) in *L. reuteri* BR11 (formerly classified as *L. fermentum* BR11) lead to defective growth in presence of oxygen with increased sensitivity to paraquat (Turner et al. 1999). The export of sulfhydryl groups and therefore the decreased ability to build a reductive environment which can exhibit a protective barrier was not given in this mutant. The role of *cyuC* after application of oxidizing conditions is suggested because an increased expression could be measured in *L. reuteri* BR11 (Hung et al. 2005). Further, higher extracellular thiol levels could be measured in the mutant compared to the wildtype (Hung et al. 2003).

1.4 Role of Mn^{2+} in the metabolism and oxidative stress response in *Lactobacillus* spp.

In the past years, Mn^{2+} gained attention because of its important role in several metabolic processes besides its function in metalloproteins. Mn^{2+}- dependent enzymes in bacteria in general are summarized in the work of Kehres & Maguire (2003). The diverse actions of Mn^{2+} in different LAB can be retrieved from BRENDA enzyme website (http://www.brenda-enzymes.org/; Schomburg et al. 2000). The detailed mode of action in carbohydrate metabolism (pyruvate oxidase, phosphofructokinase, acetate kinase, phosphoketolase and different sugar isomerases), peptide metabolism (dipeptidases, aminopeptidases) dehydrogenase reactions (malate, lactate dehydrogenase) is mainly through activation, stimulation and protection of corresponding enzymes. It is discussed that Mn^{2+} has even a distinct role in signal transduction and stabilization of the bacterial cell wall (Jakubovics & Jenkinson 2001).

In several studies it could be observed that cultures of LAB grown in media with high Mn^{2+} developed higher cell densities compared to cells with low manganese (Watanabe et al. 2012). In *E. coli* and *Salmonella typhimurium* the divalent metal transporters *Nramp* which are known for their broad substrate specificities were upregulated after application of H_2O_2, although it could be shown in *E. coli* that the imported Mn^{2+} could not effectively degrade the applied H_2O_2 (Anjem et al. 2009; Kehres et al. 2000). In *Lactococcus lactis* the inactivation of *MntH* decreased the intracellular concentration of iron and therefore OH• production from H_2O_2 via the Fenton reaction (Smith et al. 2010). During aerobiosis the manganese transporters *MntH1* and *MntH2* were expressed and increased the intracellular manganese concentration, which elevated the resistance of emerging ROS (Aguirre & Culotta 2012; Jakubovics & Jenkinson 2001; Jänsch et al. 2011). In vitro experiments revealed that Mn^{2+} reacts with superoxide to form MnO^{2+} with rapid generation of manganous phosphate, dioxygen, and H_2O_2 (Barnese et al. 2008).

The function in Mn^{2+} containing SOD and catalases was already investigated in several gram- positive bacteria (Allgood & Perry 1986; Bruno-bárcena et al. 2004; De Angelis & Gobbetti 1999; Rochat et al. 2006; Mostertz, Scharf, Hecker, Homuth, 2004). A strain of *L. plantarum* with an intracellular Mn(II) concentration of 20 to 25 mM converted emerging superoxide into H_2O_2 during aerobic conditions (Archibald & Fridovich 1981). The low intracellular Mn^{2+} concentration of *L. bulgaricus* ATCC 11842 with 0.06 mM probably accounts for the lower resistance to emerging ROS (Rochat et al. 2006; Archibald & Fridovich 1981). The protective effect of Mn^{2+} depends also on the bacterial growth phase because increased sensitivity to H_2O_2 could be detected in cells which were incubated in Mn^{2+} containing media compared to bacteria without additional Mn^{2+} (Watanabe et al. 2012). To date, the exact mechanism of $O_2^{\bullet-}$ or H_2O_2 detoxification is not fully understood.

1.5 Regulators involved in oxidative stress response in LAB

1.5.1 Peroxide- responsive repressor (*perR*)

Besides the direct actions of enzymatic and non- enzymatic mechanisms in LAB, regulators, which are activated in the presence of distinct ROS, exist in several gram- positive bacteria.

PerR, which is a metalloregulator of the *Fur* (Ferric uptake regulator) family, can specifically sense H_2O_2 by Fe^{2+} oxidation of two histidines which leads to release of Fe^{2+} and derepression of *perR* target genes as known for *Bacillus subtilis* (Lee & Helmann 2006). The binding of Mn^{2+} instead of Fe^{2+} results in formation of Mn^{2+}- *perR* complex which acts as a repressor of target genes. This *perR* regulon controls the transcription of genes mostly involved in peroxide defense. Intracellular Mn^{2+} content therefore influences the sensitivity of *perR* against H_2O_2 in *Bacillus subtilis* (Herbig & Helmann 2001).

Deletion of genes within this *perR* regulon resulted in increased peroxide sensitivity, the contrary occurred after deletion of *perR* in *Staphylococcus aureus* (Cosgrove et al. 2007). Increased resistance against H_2O_2 and cumene hydroperoxide could also be seen for a *perR* deletion mutant of *Campylobacter jejuni* (Palyada et al. 2009). It is also proposed that *ClpP* in *Staphylococcus aureus* is possibly involved in the control of transcription of members of *Fur*, *perR*, *lexA*, *MntR* and others which reveals the interconnection of proteases with metal transport and homeostasis, peroxide stress and DNA damage (Michel et al. 2006).

The information on *perR* in *Lactobacillus* spp. is still lacking. As *perR* can also be found in annotated genomes of LAB, a role in peroxide response in LAB is possible. The minor role of iron and the importance of Mn^{2+} in the metabolism of most LAB is described manifold (Archibald 1983; Elli et al. 2000; Pandey et al. 1994; Imbert & Blondeau 1998; Bruyneel et al. 1989; Archibald & Duong 1984; Archibald & Fridovich 1981; Archibald 1986; Watanabe et al. 2012), however the question if Mn^{2+} has the same effect in interacting with *perR* in LAB remains open. It is not known if a similar *perR* regulon with potential target genes for H_2O_2 detoxification exists in LAB and if the repressor activity is also influenced by H_2O_2 and Mn^{2+} in the growth media.

1.5.2 Redox- sensing transcriptional repressor (*rex*)

The redox- sensing transcriptional repressor (*rex*) which responds to the intracellular NADH/ NAD^+ levels, is involved in binding of genes involved in fermentation, glycolysis, (nitrate) respiration and biofilm formation (Bitoun et al. 2012; Brekasis & Paget 2003; Gyan et al. 2006; Pagels et al. 2010). It was studied to date in several gram- positive bacteria like *Staphylococcus aureus* (Pagels et al. 2010), *Bacillus subtilis* (Gyan et al. 2006; Wang et al. 2008), *Enterococcus faecalis* (Vesić & Kristich 2013),

Clostridium acetobutylicum (Wietzke & Bahl 2012), *Streptomyces coelicolor* (Brekasis & Paget 2003) and *Streptococcus mutans* (Bitoun et al. 2012). The protein *rex* responds if the NADH/ NAD^+ ratio is low. It represses the transcription of genes, which are involved in NADH reoxidation. The DNA-binding domain and NAD- sensing domains are highly conserved not only in the phyla *Firmicutes*. In the work of Ravcheev et al. (2012), *rex* homologs could be identified in 16 other bacterial phyla.

Deletion of *rex* leads to increased sensitivity against H_2O_2 in *Streptococcus mutans* (Bitoun et al. 2012) and even a higher accumulation in *Enterococcus faecalis* Δ*EF2638* mutant (Vesić & Kristich 2013). An increased H_2O_2 accumulation and improved growth in the presence of catalase could not be seen for the Δ*EF2933* mutant compared to the wildtype. Further, growth defects during aerobiosis, a decrease in biomass and defects in biofilm formation and increased ethanol and butanol production could be observed in *rex*- negative mutants of diverse species (Bitoun et al. 2012; Wietzke & Bahl 2012; Vesić & Kristich 2013).

1.5.3 Transcriptional regulator (*spx*)

The *spx* protein is a small and conserved protein with the characteristic CXXC motif which interacts with the α C- terminal domain of RNA polymerases to repress or activate the transcription of genes involved in different bacterial processes as known for *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus mutans* and *Enterococcus faecalis* (Liu et al. 2012; Kajfasz et al. 2010; Kajfasz et al. 2012; Nakano et al. 2003; Smith et al. 2010). As a member of the arsenate reductase (*ArsC*) family, it responds to different stressors (low pH, high temperatures, presence of bactericidal antibiotics, detergents and ROS, diamide). Interestingly in *Bacillus subtilis clpP* and *clpX* mutants had high *spx* levels. These proteases are needed for *spx* degradation (Nakano et al. 2002). Possible mechanisms include inhibition or alteration of *clpPX* activity, conformational change and thus decreased susceptibility of *spx* to *clpPX* and alterations in the structure of *spx* due to oxidation (Zuber 2004).

A *spx* deletion mutant in *Staphylococcus aureus* showed severe growth defects during non- stressing conditions (Pamp et al. 2006). In contrast to that, growth of a Δ*spx* deletion mutant in *Enterococcus faecalis* was strongly compromised at low pH, higher temperatures or in media with a high salt concentration (Kajfasz et al. 2012). Increased sensitivity could also be detected in the presence of oxygen, H_2O_2 and diamide which evidences that *spx* is involved in antioxidative mechanisms during oxidative stress. This finding is supported in *Bacillus subtilis* in which *spx* deletion lead to increased expression of methionine sulfoxide reductases A and B (*msrAB*) and extreme sensitivity to paraquat which caused modifications of the cysteine(s) in *spx* (You et al. 2008). However diamide treatment did not induce *msrAB* expression which again showed that depending on the strain and stressor, the bacterial response mechanisms differ. The activation of *spx* in *Bacillus subtilis* depends rather on the

oxidation of the cysteine residues from the CXXC motif than direct DNA binding (Nakano et al. 2005; Zuber 2004).

The function of *spx* in the control of cysteine biosynthesis genes (Choi et al. 2006) confirms that this protein is probably involved in cysteine metabolism and turnover also in other gram- positive bacteria. Actions against oxidative stress cannot be assigned to separate events alone as the "antioxidative" actions link the redox regulators with protein metabolism, DNA repair mechanisms, oxidative stress enzymes and probably proteins of which the function was overlooked so far.

1.6 Recognition motifs in proteins possibly involved in thiol- disulfide metabolism

The different roles of cysteine residues within proteins were already described. Besides its function as catalytic redox and non- redox cysteine residue, it has metal- coordinating, regulatory and structural functions and serves as site for posttranslational modifications (Fomenko et al. 2009).

Due to the high reactivity of cysteine, many thiol oxidoreductases possess besides a thioredoxin-fold, a conserved CXXC motif as outlined before (thioredoxins, glutaredoxins, redox regulators like *spx*, see chapters 1.3.2.2 and 1.5.3) in which the first cysteine acts as attacking residue and the second as resolving residue (Fomenko et al. 2009). For catalytic redox activity, the reduced SH- group is essential. Rarely, the resolving residue is replaced with serine (CXXS) or threonine (CXXT) which can also stabilize the deprotonated thiol group (Fomenko & Gladyshev 2003; Fomenko & Gladyshev 2002).

Due to the increased availability of sequenced bacterial genomes, screening for CXXC (CXXS, CXXT) motifs in protein sequences for identification of possible alternative oxidoreductases could broaden the understanding of involved proteins, which are not yet annotated and characterized. As the CXXC motif occurs also in metal- binding cysteines, structure analysis and detailed location of the motif is also important for differentiation.

It is known from *Firmicutes* that the number of proteins with disulfide bonds represents a minimum and the tendency of cysteine inclusion in exported proteins is low (Dutton et al. 2008; Daniels et al. 2010). The question remains open if the low incorporation in exported proteins and therefore possible increased intracellular incorporation of cysteine has a protective effect for LAB which lack important "antioxidative" enzymes. To date, information regarding small proteins with CXXC (CXXS, CXXT) motif and thioredoxin-like fold in LAB is insufficient.

1.7 Damages to biomolecules caused by ROS and corresponding repair mechanisms

As illustrated in Figure 1, ROS can severely damage diverse biomolecules (DNA and RNA, lipids and proteins). In the following, possible damages to proteins, DNA (RNA) and lipids are discussed with involved repair mechanisms.

1.7.1 Damages to proteins and bacterial (response and repair) mechanisms

The main protein modifications in the presence of ROS are mentioned in the review of Cabiscol et al. (2000): deficits in catalytic activity, modifications of amino acids, formation of carbonyl groups, fluorescence change, protein- protein cross- linking, oxidation of thiol groups, change in thermal stability and/ or viscosity, increased acidity, proteolysis and protein fragmentation.
LAB comprise of a protein- quality control system including chaperones and proteases, which act in folding and refolding of (damaged) proteins, prevention of protein aggregation, controlled proteolysis and others.

Stress genes in gram- positive bacteria can be classified into four groups (Narberhaus 1999; Schumann et al. 2002). Class I genes encode chaperones (*DnaK*, *GroES* and *GroEL*), which are controlled by the *HrcA* repressor. The recognition and binding of the highly conserved chaperone inverted- repeat chaperone expression (CIRCE element) sequence is inactivated during heat stress. The class II genes code for general stress proteins which are regulated by the σ^B sigma factor. Heat shock genes of class III are controlled by the *CtsR* repressor which recognizes a tandem repeat sequence. Class IV genes are not regulated through recognition sequences by *HrcA* or *CtsR* nor by σ^B sigma factor. In *Streptococcus salivarius*, dual regulation is proposed because *HrcA* and *CtsR* control *clpP* expression (Chastanet & Msadek 2003). In contrast, the expression of *clp* genes in LAB differs depending on the strain examined. In *L. plantarum clp* expression is under *CtsR*, in *L. gasseri* under *HrcA* control (Suokko et al. 2008; Fiocco et al. 2010). The role of *Clp* ATPases and proteases in processes like protein quality control, cellular differentiation, activity of transcriptional regulators (e.g. *spx*) etc. in gram- positive bacteria are summarized elsewhere (Frees et al. 2007).

The activity and mode of actions of some chaperones in LAB have been reviewed by Sugimoto et al. (2008). In LAB, known chaperones (*DnaK*, *DnaJ*, *GrpE* and *GroESL*) and proteases (*Clp*, *HtrA*, *FtsH*) are induced during heat (Suokko et al. 2008; Walker et al. 1999), acid (Lim et al. 2000; Walter et al. 2003), osmotic stress (Prasad et al. 2003) and after high pressure treatment (Hörmann et al. 2006; Pavlovic et al. 2005). The above mentioned so- called heat shock proteins and proteases are well conserved in LAB although the regulatory mechanisms are still not fully understood (Van De Guchte et al. 2002).

Oxidation of amino acid residues results mainly in formation of hydroxyl and carbonyl groups of amino acid residues, the latter is often used as marker for protein damage (Avery 2011; Mary et al. 2004). Methionine and methionine residues are most susceptible to oxidation forming methionine sulfoxides (MetSO) (Avery 2011). As these modifications lead to reduction of protein hydrophobicity and flexibility, the presence of methionine sulfoxide reductases A and B (*msrA, msrB*) enables the reduction of free and protein- bound S- and R- methionine sulfoxides (Met-S-SO, Met-R-SO) back into methionine (Ezraty et al. 2005). The reaction of *msrA* and *msrB* back into the reduced forms is accomplished using thioredoxins (Boschi-Muller et al. 2008). The observations concerning the participation of *msr* proteins during oxidative stress in bacteria are contradictory. It is known for *L. plantarum* WCFS1 that three of four *msr* genes are upregulated when the bacteria experiences coumaric acid stress (Reverón et al. 2012). In contrast, H_2O_2 treatment in *Enterococcus faecalis* did not alter *msrA* transcription (Zhao et al. 2010). H_2O_2 and paraquat lead to significant upregulation of *msrA* in *Bacillus subtilis* whereas deletion of *msrA* in *Salmonella enterica* and *Xanthomonas campestris* increased sensitivity to H_2O_2 (Denkel et al. 2011; Mostertz, Scharf, Hecker, Homuth 2004; Vattanaviboon et al. 2005). It is said that expression of *msrA* and *msrB* is growth- dependent and basal levels differ under normal physiological conditions (Romsang et al. 2013; Vattanaviboon et al. 2005).

1.7.2 Damages to DNA (RNA) and bacterial (response and repair) mechanisms

Increase in ROS can not only have deleterious effects on proteins, also DNA and RNA can be severely damaged. Possible outcomes include missing or false bases, interstrand crosslinks or strandbreaks which can lead to reconfiguration of the chromosome as described for *Bacillus subtilis* (Smith et al. 2002). Several repair enzymes with corresponding mechanisms were mainly investigated in *E. coli* (Lin & Sancar 1989). Information about detailed DNA repair mechanisms in LAB is limiting. In different *Lactobacillus* spp. exonuclease protein (*uvrA, B, C*), SOS- response regulator and protease (*lexA*), DNA recombinase (*recA*), topoisomerase (*parE, C*), ATP dependent nuclease (*addA, B*) and UV- damage repair protein (*umuC*) are the most frequently specified.

DNA double- strand breaks are mended by homologous recombination using *rec* proteins amongst others. The mechanisms of homologous recombination with participating proteins in *E. coli* have been reviewed (Kowalczykowski et al. 1994). The *recA* gene is ubiquitous which is one reason for successful application as phylogenetic marker of different bacteria and even LAB species (Torriani et al. 2001; Eisen 2011; Sarmiento-Rubiano et al. 2010). Distinct functions of *recA* include the regulation of the SOS response to DNA damage and mediation of recombination. In *Neisseria gonorrhoeae recA, recB, recC, recD, recJ, recO* and *recQ* mutants as well as holliday junction mutants *ruvA* and *ruvC* showed increased sensitivity against H_2O_2 (Stohl & Seifert 2006). A *trxB1* overexpression mutant of *L. plantarum* showed overexpression of genes involved in DNA repair (*dnaE, recA*), DNA helicases and

of polymerase *umuC*, transcriptional regulator *lexA* and stress response genes (*groESL*) when challenged with H_2O_2 (Serrano et al. 2007 b). In a Δ*clpP* mutant of *Staphylococcus aureus*, genes like *umuC*, *uvrA*, and *lexA* were upregulated connecting the actions of the *clp* proteases with DNA repair mechanisms (Michel et al. 2006).

UvrA, *B* and *C* are effective endonucleases which repair nucleotide excisions of only single bases but also intra- and interstrand crosslinks (Sancar & Rupp 1983). In *L. helveticus uvrA* which shares sequence homologies to other *uvrA* sequences in other gram- positive bacteria, repairs DNA damages after acid and H_2O_2 challenges (Cappa et al. 2005).

DNA- binding protein from starved cells (Dps) act via binding of iron or in formation of Dps- DNA complexes for protection of DNA. Dps are known for their potential to reduce the number of DNA single- strand breaks as observed for *E. coli* (Kolter 1997). The protein Dpr (for Dps-like peroxide resistance) in *Streptococcus mutans* is responsible for aerotolerance but was not able to bind DNA (Yamamoto et al. 2000). The *dpr* gene expression was downregulated in *Streptococcus thermophilus* during co- culture with *L. bulgaricus* (Sieuwerts et al. 2010). In some *Lactobacillus* spp. a gene copy of *dps* (also called DNA- binding ferritin-like protein) exists but the concrete role was not yet investigated.

1.7.3 Damages to membrane lipids and bacterial (response and repair) mechanisms

Lipids which are mainly found in the bacterial membrane can also be damaged in the presence of ROS. Formation of endogenous ROS, especially in bacteria which favor anaerobic conditions, leads to mechanisms which regulate membrane adaptation and survival (Pesakhov et al. 2007). Interconnecting the protein damage with defects in lipids could be seen in *E. coli* because the chaperonin *GroEL* stabilizes the lipid membrane, besides its function in protein folding (Török et al. 1997).

Lipid peroxidation of fatty acids can lead to aldehyde formation with impairment of typical membrane properties with a decrease in membrane fluidity. Formed aldehydes (e.g. 4-hydroxynonenal, 4-hydroxyhexenal and malonaldehyde) can react with DNA, forming aldehyde- DNA adducts causing events already described before (Marnett 2002; Meaney et al. 1990). Generation of reactive epoxides which is enhanced during low ph, oxidative and heat stress, can affect DNA and proteins (Guerzoni et al. 2001).

Fatty acids which make up 65 to 75 % of the cellular fatty acid pool in LAB were myristic (tetradecanoic; 14:0), palmitic (hexadecanoic; 16:0, hexadecenoic; 16:1), stearic (octadecanoic; 18:0), oleic, cis- vaccenic, dihydrosterculic and lactobacillic acids (Johnsson et al. 1995; Kankaanpää et al. 2004; Veerkamp 1971). Although it is proposed that most of the monounsaturated lipids in bacterial membranes are not prone for radical attack (Bielski 1983), it is evidenced that LAB can incorporate

and convert polyunsaturated fatty acids (PUFA) (Kankaanpää et al. 2004). The membrane fluidity increases with a higher content of unsaturated fatty acids, which makes it more flexible for embedded proteins and lipids. A high proportion of PUFA (C18:3) in *L. sanfranciscensis* could lead to the assumption that the strain is more prone to ROS damages (Montanari et al. 2010) compared to strains with less PUFAs.

Despite limiting information concerning the lipid damaging effect of ROS in LAB, published data suggests that potential defects and adaptation reactions in membrane fatty acids are highly strain specific but also easily influenced by environmental factors (growth media, low pH, high temperatures, osmotic differences or high pressure). In *L. hilgardii* increasing ethanol concentration favored lactobacillic acid and a decrease in oleic and vaccenic acid in the membrane (Couto et al. 1996). A low ph, high temperature and H_2O_2 treatment in *L. helveticus* lead to epoxide formation (Guerzoni et al. 2001). Higher oleic acid content after growth with linoleic and linolenic acid under acid stress favors the assumption that saturation of membrane fatty acids occurs. The proportion of medium chain fatty acid increased during acid stress in *L. sanfranciscensis*, which implicates again an increase of saturation (Montanari et al. 2010).

Besides an increase in saturation, cyclization is another mechanism to protect the intact membrane in LAB. The degree of unsaturation decreased whereas the degree of cyclization increased with higher temperatures in *L. fermentum* (Suutari & Laakso 1991). Acidification in *L. delbrueckii* subsp. *bulgaricus* caused a slight decrease of unsaturated to saturated and cyclic to saturated membrane fatty acid ratios (Streit et al. 2008). The generation of cyclopropane fatty acids (C_{19}) from oleic or cis-vaccenic acid could be found in different *L. bulgaricus* strains and were related to increased stability during cold treatment (Smittle et al. 1974). The outcomes of desaturation and cyclization in LAB are identical because the reduction of membrane fluidity prevents the passage of undesired substances (Guerzoni et al. 2001).

1.8 General information about the metabolism of *L. sanfranciscensis*

L. sanfranciscensis is the key LAB in wheat and rye sourdough and probably inhabits these food matrices since ancient times. Its dominance besides other LAB species is described in several traditionally made sourdoughs (Meroth, Walter, et al. 2003; Randazzo et al. 2005; Scheirlinck et al. 2007; Vogel et al. 1994). The genetic and phenotypic diversity of *L. sanfranciscensis* strains in sourdough is described elsewhere (Foschino et al. 2001).

The type strain *L. sanfranciscensis* DSM20451^T (other designations: ATCC 27651, NRRL B-3934) was isolated from San Francisco sourdough and is mentioned in the present work as *L. sanfranciscensis* TMW 1.53 or wildtype (WT). The majority of experiments were carried out with this strain due to its transformation ability. *L. sanfranciscensis* TMW 1.1304 was isolated from industrial sourdough fermentation in 2006. The whole genome sequence is public available since 2011 (Vogel et al. 2011). The chromosome (Accession number: NC_015978) consists of only 1,298,316 bp with two additional plasmids, *pLS1* (Accession number: NC_015979) and *pLS2* (Accession number: NC_015980), with sizes of 58,739 bp and 18,715 bp (Vogel et al. 2011). The strain has the highest rRNA operon density (5.39 per Mbp) among so far known free- living organisms (Vogel et al. 2011).

The main physiology, interactions with yeasts and genetic aspects of *L. sanfranciscensis* were already reviewed (Gobbetti & Corsetti 1997). This obligate heterofermentative bacterium exhibits an effective maltose metabolism which is highly preferred compared to glucose which clearly explains its occurrence in the narrow niche like cereal- based fermentation. The characteristic phosphorylitic cleavage of maltose with preferred glucose export was already discussed elsewhere (Neubauer et al. 1994) and key enzymes maltosephosphorylase (*mapA*) and phosphoglucomutase (*pgmA*) have been characterized (Ehrmann & Vogel 1998).

Further, it is known that the strain can use fructose, oxygen, citrate, pyruvate and α-ketoglutarate as external electron acceptors, which increases growth rate and cell yield (Stolz et al. 1995; Zhang & Gänzle 2010). For example, externally added pyruvate is reduced to lactate with reoxidation of NADH. Malate is not used as electron acceptor by *L. sanfranciscensis*. In the presence of external electron acceptors, acetate is formed instead of ethanol via acetate kinase (*ack*) reaction with formation of an additional ATP as already outlined in chapter 1.1 (Knorr, Ehrmann 2001). The use of fructose and oxygen as external electron acceptors has been well studied in *L. sanfranciscensis* as visible in Figure 2. The enzymes phosphotransacetylase (*pta*), acetate kinase (*ack*) and *nox* are involved in acetate formation. The production of acetate instead of ethanol forms ATP but does not regenerate coenzymes which are important to balance the electron flow.

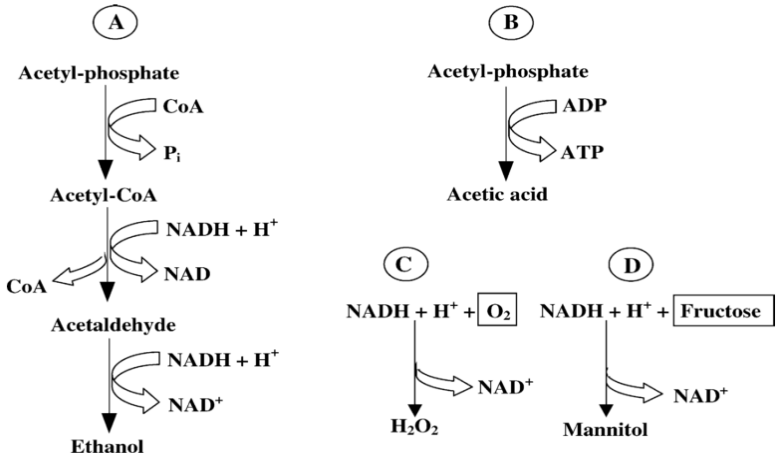


Figure 2: Use of fructose and oxygen as external electron acceptors by *L. sanfranciscensis.* (A) Two- carbon branch of the phosphogluconate pathway; **(B)** acetate kinase reaction; **(C)** use of oxygen; and **(D)** fructose as external electron acceptors. Picture taken from (Gobbetti et al. 2005).

Proteolytic activity is not very common among LAB found in sourdough and strain specific. No proteolytic activity is described for *L. sanfranciscensis* DSM 20451^T (Vermeulen et al. 2005), whereas the metabolism of amino acids depends on peptide availability and hydrolysis activity. In sourdough, exponentially growing bacterial cells express peptide transporter (*opp*, *dtpP*) and peptidase genes (*pepN*, *pepC*, and *pepT*) for peptide uptake and breakdown which originate from endogenous flour proteinases (Vermeulen et al. 2005). Based on the genome information of *L. sanfranciscensis* TMW 1.1304, the synthesis of four amino acids (alanine from pyruvate, aspartate from oxaloacetate, glutamate and glutamine) can be accomplished whereas the strain is auxotroph for the other twelve amino acids (Vogel et al. 2011). The high adaptation of *L. sanfranciscensis* to protein- rich wheat and rye dough with its low concentration of free amino acids and the strains preference for peptide import and intracellular turnover yielding amino acids was clearly evidenced.

1.9 *L. sanfranciscensis* and oxidative stress

L. sanfranciscensis as obligate heterofermentative LAB lacks the respiratory chain but is able to use oxygen as external electron acceptor resulting in a higher final cell yield and growth rate compared to anaerobic growth conditions (Stolz et al. 1995; De Angelis & Gobbetti 1999; Jänsch et al. 2011). The presence of genes in the genome of *L. sanfranciscensis* TMW 1.1304, which could be involved in the resistance against oxidative stress can be summarized (Vogel et al. 2011):

The presence of NADH oxidase 2 (*nox*-2; LSA_05610) catalyzes the four- electron reduction of oxygen to water (Riebel et al. 2003). The essentiality of this enzyme during aerobic incubation with increase in energy gain was already discussed. A Δ*nox* mutant of *L. sanfranciscensis* DSM20451^T

showed increased sensitivity during aerobiosis and in the presence of paraquat and diamide in MRS media without additional electron acceptors (Jänsch et al. 2011). The mutant failed in regeneration of NADH during aerobic incubation which could be shown in a decreased acetate/ lactate ratio.

For detoxification of superoxide, a Mn^{2+}- containing *sodA* exists in *L. sanfranciscensis* CB1 and it could be observed that aerobic incubation together with Mn^{2+} in MRS media lead to cell death after reaching the stationary phase (De Angelis & Gobbetti 1999). The strain accumulated high amounts of H_2O_2 due to *sodA* activity (and probably NADH oxidase 1). No *sodA* homologue could be found in the genome of *L. sanfranciscensis* TMW 1.1304.

L. sanfranciscensis lacks the enzymes catalase, *ahp* and *npx*, thus the elimination of H_2O_2 can only be executed by free available Mn^{2+}. The role of manganese in LAB was discussed in chapter 1.4.

In *L. sanfranciscensis* TMW 1.1304 two genome copies of thioredoxin reductases (LSA_02530; LSA_05170), one genome copy of thioredoxin (*trxA*, LSA_08950) and three thioredoxin-like proteins (LSA_08950, LSA_02610, LSA_06080) exist.

The synthesis of GSH is unfeasible due to the absence of enzymes like γ-glutamyl cysteine synthetase (*gshA*), glutathione synthetase (*gshB*) or glutathione biosynthesis bifunctional fusion gene (*gshA/B/ gshF*). However the presence of glutathione reductase (*gshR*/ *gor*, LSA_2p00270) and glutathione peroxidase (*gpo/ gpx*, LSA_09790) suggest an essential role of GSH in *L. sanfranciscensis*. *GshR* reduces GSSG to two GSH monomers with simultaneous oxidation of NADH. In *L. sanfranciscensis* GshR is responsible for an increase in thiol levels in sourdough. A deletion of the gene decreased thiol levels and affected also the resistance against oxidative stress (Jänsch et al. 2007).

Other genes which are involved in actions against oxidative stress in *L. sanfranciscensis* TMW 1.1304 include the glutaredoxin-like protein (*nrdH*, LSA_04700) besides *nrdE, F, R* and a putative *nrdI*-like protein. The presence of *msrA* (LSA_07350 and *msrB* (LSA_07360) enables the strain to reduce oxidized methionine.

Regulators can also be found in the genome of *L. sanfranciscensis*. Peroxide stress can be detected by different sensors in bacteria. The following sensors and regulators can be found in the annotated genome of *L. sanfranciscensis*: *ohrR* (LSA_05940), *perR* (LSA_03000), regulatory protein *spxA* (LSA_02420) and redox- sensing transcriptional repressor *rex* (LSA_04930). The role of *perR*, *spx* and *rex* in LAB in general has already been explained in chapter 1.5. *OhrR* is a transcriptional repressor with cysteine residues which are oxidized in the presence of organic peroxides. The repression of *ohr* target genes follows to detoxify organic hydroperoxides (not H_2O_2). The role was already characterized in *Pseudomonas aeruginosa* (Atichartpongkul et al. 2010), *Streptomyces coelicolor* (Oh et al. 2007), *Xanthomonas campestris* pv. *phaseoli* (Panmanee et al. 2006) and others. As *ohr* target genes are absent in the genome of *L. sanfranciscensis* and the gene is labeled as "pseudogene", a possible role of *ohrR* in *L. sanfranciscensis* is questionable.

The annotation of a peroxide- responsive repressor *perR* (LSA_03000) reflects that this strain probably possesses a peroxide sensor. The protein consists of two CXXC motifs however target genes which are regulated by *perR* are unknown. In *L. sanfranciscensis* TMW 1.1304 one genome copy of *rex*, which consists of 216 amino acids can be found. A blastp search indicates high sequence similarity to LAB and other gram- positive bacteria. One genome copy of the regulatory protein *spxA* (LSA_02420) is present in *L. sanfranciscensis*.

Genes coding for chaperones and proteases are also present in the genome of *L. sanfranciscensis*. The role of proteases like *clpP* (LSA_05260), *clpX* (LSA_09640), *clpE* (LSA_10410), *clpC* (LSA_12680) and *ftsH* (LSA_04310) which are involved in diverse reactions against oxidative stress in diverse LAB were already mentioned. Chaperone like *groS* (LSA_04980), *groL* (LSA_04990), *dnaJ* (LSA_08110), *dnaK* (LSA_08120) and *grpE* (LSA_08130) can be retrieved from annotation. In the genome of *L. sanfranciscensis* both regulators *hrcA* (LSA_08140) and *ctsR* (LSA_11440) can be found which proposes a dual regulation as known for *Streptococcus salivarius* (Chastanet & Msadek 2003).

Present DNA repair proteins include: *recF* (LSA_00040), *recN* (LSA_06620), *recO* (LSA_07760), *radA*-like protein (LSA_04490) and DNA mismatch repair proteins *mutL* (LSA_09080) and *mutS* (LSA_09090). *UvrA* (LSA_05190), *uvrB* (LSA_05180) and *uvrC* (LSA_09610) are known to encode exinuclease proteins. *RecA* (LSA_01160), *recN* (LSA_06620), *recO* (LSA_07760) are also known to be involved in DNA repair. Others include topoisomerases like *parC* (LSA_07380), *parE* (LSA_07390), nuclease *addA* (LSA_13260), SOS response regulator *lexA* (LSA_08380) and ATP-dependent DNA helicases *ruvA* (LSA_09070) and *ruvB* (LSA_09060). Under stress conditions these genes encoding proteins, which are induced after DNA damage or blocking of DNA replication.

2 HYPOTHESIS AND AIMS

Previous work in *L. sanfranciscensis* DSM20451^T showed that the redox active enzymes glutathione reductase (*gshR*) and NADH oxidase (*nox*) involved in redox homeostasis having a significant role in allocation of reducing thiol equivalents (*gshR*) and generation of NAD^+ during aerobic incubation (*nox*).

In order to improve the understanding of not yet described genes involved in redox reactions ("redox genes") in *L. sanfranciscensis*, the role of thioredoxin reductase (*trxR*), redox- sensing repressor (*rex*), glutathione peroxidase (*gpo, gpx*) and cystine transport permease (*tcyB*) should be highlighted in this work. It is hypothesized that these enzymes influence the intracellular redox homeostasis in *L. sanfranciscensis* especially in oxidative environments.

It is proposed that TrxR is essential to keep the intracellular homeostasis in a reduced state. Rex is generally described as regulator of the intracellular NAD: NADH ratio, and may act so also in *L. sanfranciscensis*, i.e. influencing the metabolic status of the cell. Gpo probably detoxifies emerging H_2O_2 and therefore protects the bacterium from further damage. Furthermore, TcyB is possibly involved in maintenance of the intracellular thiol homeostasis and resistance against ROS.

It is expected that especially under aerobic conditions where ROS formation occurs, generally growth of deletion mutants is impaired. The application of oxidative substances like paraquat (superoxide stress), diamide (thiol stress) and H_2O_2 (peroxide stress) should therefore give first information if the deleted genes participate in actions caused by superoxide, thiol and peroxide stress. The measurement of ORP including metabolite analysis, the determination of (intracellular and extracellular) thiol groups and H_2O_2 quantification are applied to get further information about the genes' participation during intracellular redox reactions in this bacterium.

Another part of the work should include screening for the presence and distribution of known "redox genes" in other LAB with degenerated primers. Based on this, groupings should be conducted and growth with oxidative agents should give information, if there is a connection between presence of "antioxidative" genes and their physiological tolerance against oxidants. It is hypothesized that strains with a larger set of "redox genes" and therefore an increased "antioxidative" potential tolerate the presence of oxidative agents like H_2O_2 and diamide much better.

The final aim was to determine the role of other, so far unknown "redox genes", which are involved in the resistance against specific environmental stressors [presence of electron acceptors like oxygen and fructose, co- cultivation with *Candida (C.) humilis*] in *L. sanfranciscensis*. For that purpose microarray analysis, RNA sequencing and *in-silico* protein prediction analysis should be used.

3 MATERIAL AND METHODS

3.1 Material

3.1.1 Devices

Major devices used in this work are listed in Table 1 in alphabetical order.

Table 1: Devices used in this study.

Device	Model	Manufacturer
Agarose gel chamber 25 x 20 cm	Easy Cast electrophoresis system	Owl Separation Systems, Portsmouth, NH, USA
Agarose gel chamber 13.8 x 12 cm	Easy Cast electrophoresis system	Owl Separation Systems, Portsmouth, NH, USA
Autoclaves	VE- 40	Systec GmbH, Wettenberg, Germany
	VX- 150	Systec GmbH, Wettenberg, Germany
	Varioklav	H + P Labortechnik, Oberschleißheim, Germany
Breeding/incubation	Certomat BS- 1	B. Braun Biotech International, Melsungen, Germany
	Hereaus B5042E	Hereaus Instruments, Hanau, Germany
	Memmert INB series	Memmert GmbH & Co. KG, Schwabach, Germany
	WiseCube®WIS- ML02	Witeg Labortechnik GmbH, Wertheim, Germany
Centrifuges	Sigma 1 K 15	Sigma Labortechnik, Osterrode am Harz, Germany
	Sigma 6- 16K	Sigma Labortechnik, Osterrode am Harz, Germany
	J- 6	Beckman, Palo alto, CA, USA
	J- 2	Beckman, Palo alto, CA, USA
	HermLe Z383 K	HermLe Labortechnik, Wehningen, Germany
	HermLe Z382 K	HermLe Labortechnik, Wehningen, Germany
Drying machine	Venticell	MMM Medcenter Einrichtungen GmbH, Planegg, Germany
Electroporation system	Bio- Rad Gene pulser device	Bio- Rad Laboratories, Hercules, CA, USA
Fast Prep® System		MP Biomedicals, Solon, USA
Fermenter 3x 500 mL	Biostat®Q	B. Braun Biotech International, Melsungen, Germany
Ice mahine	AF100	Scotsman®, Milan, Italy
Incubation hood	Certomat H	B. Braun Biotech International, Melsungen, Germany
Ionenchromatography	ICS 5000	Dionex, Sunnyvale, USA
Laminar flow sterile work bench	HERA safe	Heraeus Instruments, Hanau, Germany
Microscope	Axiolab	Carl Zeiss MicroImaging GmbH, Germany
Microwave	intellowave	LG, Seoul, South Corea
Nanodrop	Nanodrop1000	Peqlab Biotechnologie GmbH, Erlangen, Germany
PCR- Cycler	Primus 96 plus	MWG Biotech, AG, Ebersberg, Germany
	Mastercycler gradient	Eppendorf AG, Hamburg, Germany
pH determination (electrode)	InLab 412, pH 0- 14	Mettler- Toledo, Gießen, Germany
pH determination (fermenter)	ProLab3000	Schott® Instruments
Pipettes	Pipetman	Gilson- Abomed, Langenfeld, Germany

Device	Model	Manufacturer
Plate readers	TECAN SPECTRAFluor	TECAN Deutschland GmbH, Crailsheim, Germany
	FLUOstar Omega	BMG Labtech, Ortenberg, Germany
	TECAN SUNRISE	TECAN Deutschland GmbH, Crailsheim, Germany
Plating machine Eddy Jet		IUL Instruments, Königswinter, Germany
Power supplies	MPP 2 x 3000 Power Supply	MWG Biotech AG, Ebersberg, Germany
	Electroophoresis Power Supply EPS 3000	Pharmacia Biotech, Cambridge, England
	2197 Supply PPS 200- 1D	MWG Biotech AG, Ebersberg, Germany
Scales	SPD 61, SBA 52	Scaltec, Dania, USA
Shaking	Certomat R	B. Braun Biotech International, Melsungen, Germany
	Vortex 2 Genie	Scientific Industries Inc., Bohemia, NY, USA
Stirring	RCT- Basic	Mettler- Toledo, Gießen, Germany
Thermo block	Techne DRI- Block DB3	Thermo- Dux Gesellschaft für Laborgerätebau mbH, Wertheim, Germany
Ultra sonic water bath	Sonorex Super RK 103H	Bandelin electronic, Berlin, Germany
UV table	Herolab UVT 28M	Herlab GmbH Laborgeräte, Wiesloch, Germany
Water bath	Lauda BD	LAUDA Dr. D. Wobser GmbH & Co., Lauda-Königshofen, Germany

3.1.2 Chemicals

Chemicals used in this work are listed in Table 2 in alphabetical order.

Table 2: Chemicals used in this study.

Chemicals	Purity	Manufacturer
6 x DNA loading dye	-	Fermentas GmbH, St. Leon- Rot, Germany
Acetic acid	99 - 100 % (glacial)	Merck, Darmstadt, Germany
Acetone	for HPLC	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Adenine	≥99 %	SIGMA- Aldrich, Steinheim, Germany
Agar	european agar	Difco, BD Sciences, Heidelberg
Agarose	for electrophoresis	Biozym Scientific GmbH, Oldendorf, Germany
L- Alanine	≥98 %	SIGMA- Aldrich, Steinheim, Germany
Amino acids	research grade	SERVA, Heidelberg, Germany
Ampicillin sodium salt	93.3 %	Gerbu Biotechnik GmbH, Gaiberg, Germany
Ammonium chloride	≥99.5 % p.a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
L- Arginine	-	Merck, Darmstadt, Germany
L- Asparagine	≥98 %	SIGMA- Aldrich, Steinheim, Germany
Biotin	~98 %	SIGMA- Aldrich, Steinheim, Germany
Bromcresol purple	p.a.	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Bromphenol blue	for electrophoresis	SIGMA- Aldrich, Steinheim, Germany
Cysteine hydrochloride * H_2O	p.a.	Roth, Arlesheim, Germany
Cystine	≥98 %	SIGMA- Aldrich, Steinheim, Germany
Dimidium bromide	≥98 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Diamide	-	SIGMA- Aldrich, Steinheim, Germany

Chemicals	**Purity**	**Manufacturer**
DMSO (dimethyl sulfoxide)	≥99.5 % p. a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
DTNB (Ellmans Reagenz)	≥98 %	SIGMA- Aldrich, Steinheim, Germany
DTT (1,4 Dithio- D,L- Threitol)	high purity	GERBU Biotechnik, GmbH, Gaiberg, Germany
EDTA	for molecular biology	GERBU Biotechnik, GmbH, Gaiberg, Germany
Erythromycin base	-	SERVA Electrophoresis GmbH, Heidelberg, Germany
Ethanol, denatured	99 % with 1 % methylethylketone	Chemikalien und Laborbedarf Nierle, Freising, Germany
Ethanol, absolute	≥99.8 %	VWR, Prolabo, Foutenay- sous- Bois, France
Ethidium bromide	1 % in H_2O for electrophoresis	Merck, Darmstadt, Germany
Fast- AP	-	Fermentas GmbH, St. Leon- Rot, Germany
FD restriction buffer	-	Fermentas GmbH, St. Leon- Rot, Germany
FD restriction enzymes	-	Fermentas GmbH, St. Leon- Rot, Germany
FeSO4*7H2O		SIGMA- Aldrich, Steinheim, Germany
Folic acid	p. a.	SIGMA- Aldrich, Steinheim, Germany
Fructose		OMNI Life Science, Bremen, Germany
Glucose	for biochemical use	Merck, Darmstadt, Germany
Glutathion, reduced		SIGMA- Aldrich, Steinheim, Germany
Glycerol	99.5 %, high purity	GERBU Biotechnik, GmbH, Gaiberg, Germany
Glycine	p. a.	GERBU Biotechnik, GmbH, Gaiberg, Germany
Guanine	≥99.8 %	Merck, Darmstadt, Germany
HCl 37 %	p.a.	Merck, Darmstadt, Germany
Histidine monohydrochloride	-	Merck, Darmstadt, Germany
Horse radish peroxidase		AppliChem, Gattersleben, Germany
Hydrogen peroxide	30 %	Merck, Darmstadt, Germany
IPTG	p.a.	GERBU Biotechnik, GmbH, Gaiberg, Germany
Isoleucine	-	SERVA, Heidelberg, Germany
Isopropanol	p.a.	Scharlau Chemi S. A., Sentmenat, Spain
KH_2PO_4	p. a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
K_2HPO_4 * 3 H_2O	p.a.	Merck, Darmstadt, Germany
Kalium acetate	p. a.	Merck, Darmstadt, Germany
KCl	p. a.	Merck, Darmstadt, Germany
L- amino acids	p.a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Leucine	-	Merck, Darmstadt, Germany
Lysozyme	-	SERVA, Heidelberg, Germany
Maltose	Biochemical grade	GERBU Biotechnik GmbH, Heidelberg, Germany
Meat extract	for microbiology	Merck, Darmstadt, Germany
Methanol	HPLC- grade	Mallinkrodt Baker B. V., Deventer, NL
Methionine	≥99 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
$MgCl_2$ * 6 H_2O	p.a.	SIGMA- Aldrich, Steinheim, Germany

Chemicals	**Purity**	**Manufacturer**
$MgSO_4$ * 7 H_2O	p.a.	Merck, Darmstadt, Germany
$MnCl_2$ * 4 H_2O	p.a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
$MnSO_4$ * 4 H_2O	p.a.	Merck, Darmstadt, Germany
NaCl	p.a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
NaH_2PO_4	p.a.	Merck, Darmstadt, Germany
NaOH	p.a.	Merck, Darmstadt, Germany
Nicotinic acid	p.a.	SIGMA- Aldrich, Steinheim, Germany
Ninhydrin	p.a.	Merck, Darmstadt, Germany
Orotic acid	p.a.	SIGMA- Aldrich, Steinheim, Germany
p- amino benzoic acid	≥99.8 %	SIGMA- Aldrich, Steinheim, Germany
Panthothenic acid	p.a.	SIGMA- Aldrich, Steinheim, Germany
Paraffin oil	-	SIGMA- Aldrich, Steinheim, Germany
Perchloric acid	70 %	SIGMA- Aldrich, Steinheim, Germany
Pepton from casein	for microbiology	Merck, Darmstadt, Germany
Phenylalanine	≥98 %	SIGMA- Aldrich, Steinheim, Germany
Phusion® DNA Polymerase	-	New England Biolabs, County Road, USA
Pyridoxal 5´- phosphate hydrate	-	SIGMA- Aldrich, Steinheim, Germany
Primer	-	MWG- BiotechAG, Ebersberg, Germany
Proline	≥99 %	SIGMA- Aldrich, Steinheim, Germany
Pyridoxal- HCl	p.a.	SIGMA- Aldrich, Steinheim, Germany
RINGER solution	for microbiology	Merck, Darmstadt, Germany
SDS	research grade	SERVA, Heidelberg, Germany
L- Serine	≥99 %	SIGMA- Aldrich, Steinheim, Germany
Sodium acetate * 3 H_2O	p.a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium azide	p.a.	SIGMA- Aldrich, Steinheim, Germany
Sodium citrate	p.a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium hydroxide	for HPLC	JT Baker, Griesheim, Germany
Sodium phosphate	p.a.	Merck, Darmstadt, Germany
Sucrose	HPLC- grade	GERBU Biotechnik GmbH, Heidelberg, Germany
Sulfuric acid	p.a.	Merck, Darmstadt, Germany
T4 DNA ligase	-	Fermentas GmbH, St. Leon- Rot, Germany
Taq Core Kit	-	MP Biomedicals Solon, Ohio, USA
Tetramethylbenzidin	≥98 %	AppliChem, Gattersleben, Germany
Thiamine HCl	-	SIGMA- Aldrich, Steinheim, Germany
L- Threonine	≥98.5 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Tris	ultra pure	MP Biomedicals Solon, Ohio, USA
Tris base	ultra pure	ICN Biomedicals, Inc., Ohio, USA
Tris- HCl	p.a.	Merck, Darmstadt, Germany
L- Tryptophane	≥98.5 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Tween 80	-	Mallinkrodt Baker B. v., Deventer, NL

Chemicals	Purity	Manufacturer
L- Tyrosine	-	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Uracil	p.a.	SIGMA- Aldrich, Steinheim, Germany
L- Valine	≥98 %	SIGMA- Aldrich, Steinheim, Germany
Xanthine	p.a.	SIGMA- Aldrich, Steinheim, Germany
X- Gal (5- Bromo- 4- chloro- 3- indolyl- ß- D- galactobpyranoside)	p.a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Xylene Cyanol FF	-	SIGMA- Aldrich, Steinheim, Germany
Yeast extract	for microbiology	Merck, Darmstadt, Germany
ZnSO4	≥ 99.5 %	SIGMA- Aldrich, Steinheim, Germany

3.1.3 Expendable materials

Other materials used in this work are listed in Table 3 in alphabetical order.

Table 3: Further materials used in this study.

Material	Type	Manufacturer
Anaerocult C mini	-	Merck, Darmstadt, Germany
Combitips	0.5 mL; 2.5 mL; 10 mL	Eppendorf, Hamburg, Germany
Electroporation cuvettes	-	Biozym scientific GmbH, Oldendorf, Germany
		MP Biomedicals, Solon, USA
Fast Prep 24 Lysis Matrix tubes	0.1 mm Silica spheres	
Microtiter plates	multi well plate 96- well flat bottom with lid	Sarstedt, Nümbrecht, Germany
Reaction tubes	2 mL, 1.5 mL, 200 µL	Eppendorf, Hamburg, Germany
Sterile mL tubes	5 mL, 15 mL, 50 mL	Sarstedt, Nümbrecht, Germany
Sterile filter	Filtropur S 0.2 (0.2 µm)	Sarstedt, Nümbrecht, Germany
Sterile sensi- discs	Blank (no antibiotic)	BD Diagnostics, Heidelberg, Germany
Anaerocult	-	Merck, Darmstadt, Germany

3.1.4 Kits

Kits used in this work are listed in Table 4 in alphabetical order.

Table 4: Kits used in this study.

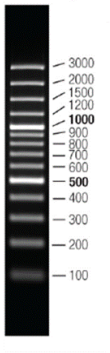
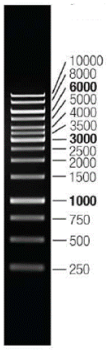
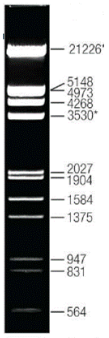
Kit	Type	Manufacturer
E.Z.N.A. Bacterial DNA Kit	DNA isolation	Omega Bio- Tek Inc., Norcross, GA, USA
E.Z.N.A. Plasmid Mini Kit II		Omega Bio- Tek Inc., Norcross, GA, USA
		Roche, Basel, Switzerland
High Pure RNA isolation	RNA extraction	Qiagen GmbH, Hilden, Germany
QIAprep®spin Miniprep Kit	Plasmid extraction	
Pierce Quantitative Peroxide Assay Kit	Lipid	Thermo Fisher Scientific, Waltham, MA, USA
Qiagen® Plasmid Plus Midi Kit	Plasmid extraction	Qiagen GmbH, Hilden, Germany

Kit	Type	Manufacturer
QIAquick PCR purification Kit	PCR purification Kit	Qiagen GmbH, Hilden, Germany
RNeasy® Midi Kit	RNA extraction	Qiagen GmbH, Hilden, Germany
Taq Core Kit	DNA polymerase	MP Biomedicals Solon, Ohio, USA

3.1.5 DNA markers

DNA markers used for size estimations were purchased from Fermentas GmbH, St. Leon- Rot and are listed in Table 5.

Table 5: DNA markers used in this study.

GeneRuler™ 100 bp DNA Ladder	**GeneRuler™ 1 kb DNA Ladder**	**Lambda DNA/*EcoRI+HindIII* Marker, 3**
		

3.1.6 Bacterial strains

All strains used in this work were obtained from TMW culture collection, stored at – 80 °C as glycerol stocks (see Table 6). For knock- out and cloning experiments, *E. coli* strains DH5α and TOP 10 were used.

Table 6: Bacterial strains used for PCR screening in this work including abbreviations, origin of isolation, fermentation type and growth temperature.

no.	**strains**	**abbreviations**	**origin of isolation**	**metabolism**	**temperature [°C]**
1	*Enterococcus (E.) faecalis* TMW 2.630	E. fa.	sheep cheese	homofermentative	37
2	*Lactobacillus (L.) acidophilus* TMW 1.18	L. acid.	DSM 20079^T	obligate homofermentative	37
3	*Lactobacillus (L.) brevis* TMW 1.100	L. brev.	sourdough	heterofermentative	30
4	*Lactobacillus (L.) brevis* TMW 1.1785	L. brev.	rice sourdough	heterofermentative	30
5	*Lactobacillus (L.) brevis* TMW 1.1786	L. brev.	rice sourdough	heterofermentative	30
6	*Lactobacillus (L.) brevis* TMW 1.1787	L. brev.	rice sourdough	heterofermentative	30
7	*Lactobacillus (L.) brevis* TMW 1.1807	L. brev.	fermented food	obligate homofermentative	30
8	*Lactobacillus (L.) brevis* TMW 1.6	L. brev.	DSM 20054^T	obligate homofermentative	30
9	*Lactobacillus (L.) brevis* TMW 1.1326	L. brev.	ATCC 367	obligate homofermentative	30
10	*Lactobacillus (L.) brevis* TMW 1.313	L. brev.	beer	obligate homofermentative	30
11	*Lactobacillus (L.) brevis* TMW 1.1369	L. brev.	honey	obligate homofermentative	30
12	*Lactobacillus (L.) brevis* TMW 1.57	L. brev.	DSM 20054^T	obligate homofermentative	30

no.	**strains**	**abbreviations**	**origin of isolation**	**metabolism**	**temperature [°C]**
13	*Lactobacillus (L.) brevis* TMW 1.791	L. brev.	DSM 20556	obligate homofermentative	30
14	*Lactobacillus (L.) brevis* TMW 1.384	L. brev.	sourdough	obligate homofermentative	30
15	*Lactobacillus (L.) casei paracasei* TMW 1.1462	L. c. par.	sourdough	facultative heterofermentative	30
16	*Lactobacillus (L.) curvatus* TMW 1.624	L. curv.	raw sausage	facultative heterofermentative	30
17	*Lactobacillus (L.) delbrueckii subsp. delbr.* TMW 1.58	L. delbr.	DSM 20074^{T}	obligate homofermentative	37
18	*Lactobacillus (L.) farciminis* TMW 1.68	L. farc.	DSM 20184^{T}	obligate homofermentative	30
19	*Lactobacillus (L.) fermentum* TMW 1.1788	L. ferm	rice sourdough	obligate heterofermentative	30
20	*Lactobacillus (L.) fermentum* TMW 1.1837	L. ferm	fermented diary	obligate heterofermentative	30
21	*Lactobacillus (L.) fermentum* TMW 1.1836	L. ferm	fermented diary	obligate heterofermentative	30
22	*Lactobacillus (L.) fermentum* TMW 1.1835	L. ferm	fermented diary	obligate heterofermentative	30
23	*Lactobacillus (L.) fermentum* TMW 1.1727	L. ferm	rice sourdough	obligate heterofermentative	30
24	*Lactobacillus (L.) graminis* TMW 1.1174	L. gra.	grass silage(DSM 20719)	facultative heterofermentative	30
25	*Lactobacillus (L.) mindensis* TMW 1.1206	L. min.	sourdough (DSM 14500)	heterofermentative	30
26	*Lactobacillus (L.) panis* TMW 1.648	L. pan.	DSM 6035	obligate heterofermentative	37
27	*Lactobacillus (L.) paracasei* TMW 1.1305	L. par.	sourdough	facultative heterofermentative	30
28	*Lactobacillus (L.) paracasei* TMW 1.1434	L. par.	milk	facultative heterofermentative	30
29	*Lactobacillus (L.) paracasei* TMW 1.1724	L. par.	sourdough	facultative heterofermentative	30
30	*Lactobacillus (L.) paracasei* TMW 1.304	L. par.	beer	facultative heterofermentative	30
31	*Lactobacillus (L.) paracasei subsp. paracasei* TMW 1.1213	L. par. par.	sourdough	facultative heterofermentative	30
32	*Lactobacillus (L.) paralimentarius* TMW 1.1234	L. paralim.	sourdough	facultative heterofermentative	30
33	*Lactobacillus (L.) paralimentarius* TMW 1.1235	L. paralim.	sourdough	facultative heterofermentative	30
34	*Lactobacillus (L.) paralimentarius* TMW 1.1726	L. paralim.	rice sourdough	facultative heterofermentative	30
35	*Lactobacillus (L.) paralimentarius* TMW 1.256	L. paralim.	sourdough (DSM 13238)	facultative heterofermentative	30
36	*Lactobacillus (L.) paralimentarius* TMW 1.1725	L. paral.	sourdough	facultative heterofermentative	30
37	*Lactobacillus (L.) perolens* TMW 1.500	L. perol.	unknown	facultative heterofermentative	30
38	*Lactobacillus (L.) plantarum* TMW 1.1204	L. pla	sourdough (DSM 13238)	facultative heterofermentative	30
39	*Lactobacillus (L.) plantarum* TMW 1.1237	L. pla	sourdough	facultative heterofermentative	30
40	*Lactobacillus (L.) plantarum* TMW 1.124	L. pla	sourdough	facultative heterofermentative	30
41	*Lactobacillus (L.) plantarum* TMW 1.1723	L. pla	sourdough	facultative heterofermentative	30
42	*Lactobacillus (L.) plantarum* TMW 1.460	L. pla	sourdough	facultative heterofermentative	30
43	*Lactobacillus (L.) plantarum* TMW 1.702	L. pla	sourdough	facultative heterofermentative	30
44	*Lactobacillus (L.) plantarum* TMW 1.1	L. pla	raw sausage	facultative heterofermentative	30
45	*Lactobacillus (L.) plantarum* TMW 1.1732	L. pla	fermented food	facultative heterofermentative	37
46	*Lactobacillus (L.) plantarum* TMW 1.701	L. pla	sourdough	facultative heterofermentative	30
47	*Lactobacillus (L.) plantarum* TMW 1.1372	L. pla	honey	facultative	30

no.	**strains**	**abbreviations**	**origin of isolation**	**metabolism**	**temperature [°C]**
				heterofermentative	
48	*Lactobacillus (L.) plantarum* TMW 1.1478	L. pla	honey	facultative heterofermentative	30
49	*Lactobacillus (L.) plantarum* TMW 1.1809	L. pla	fermented food	facultative heterofermentative	37
50	*Lactobacillus (L.) pontis* TMW 1.56	L. pon.	rye sourdough	obligate heterofermentative	37
51	*Lactobacillus (L.) pontis* TMW 1.1300	L. pon.	sourdough	obligate heterofermentative	30
52	*Lactobacillus (L.) pontis* TMW 1.1301	L. pon.	sourdough	obligate heterofermentative	30
53	*Lactobacillus (L.) pontis* TMW 1.1086	L. pon.	rye sourdough DSM 8475^T	obligate heterofermentative	37
54	*Lactobacillus (L.) reuteri* TMW 1.106	L. reut.	sourdough	obligate heterofermentative	40
55	*Lactobacillus (L.) sakei* TMW 1.704	L. sak.	sourdough	facultative heterofermentative	30
56	*Lactobacillus (L.) sakei* TMW 1.705	L. sak.	sourdough	facultative heterofermentative	30
57	*Lactobacillus (L.) sakei* TMW 1.1239	L. sak.	sourdough	facultative heterofermentative	30
58	*Lactobacillus (L.) sanfranciscensis* TMW 1.53	L. sanfr.	sourdough DSM 20451^T	obligate heterofermentative	30
59	*Lactobacillus (L.) sanfranciscensis* TMW 1.728	L. sanfr.	-	obligate heterofermentative	30
60	*Lactobacillus (L.) sanfranciscensis* TMW 1.392	L. sanfr.	-	obligate heterofermentative	30
61	*Lactobacillus (L.) sanfranciscensis* TMW 1.377	L. sanfr.	sourdough DSM 20451^T	obligate heterofermentative	30
62	*Lactobacillus (L.) sanfranciscensis* TMW 1.398	L. sanfr.	sourdough	obligate heterofermentative	30
63	*Lactobacillus (L.) sanfranciscensis* TMW 1.1461	L. sanfr.	sourdough	obligate heterofermentative	30
64	*Lactobacillus (L.) sanfranciscensis* TMW 1.1304	L. sanfr.	sourdough	obligate heterofermentative	30
65	*Lactobacillus (L.) spicheri* TMW 1.1226	L. spich.	sourdough	obligate heterofermentative	30
66	*Lactobacillus (L.) spicheri* TMW 1.1233	L. spich.	sourdough	obligate heterofermentative	30
67	*Lactobacillus (L.) spicheri* TMW 1.1225	L. spich.	sourdough	obligate heterofermentative	30
68	*Leuconostoc (Leuc.) holzapfelii* TMW 2.813	Leuc. holz.	-	obligate heterofermentative	30
69	*Pediococcus (P.) pentosaceus* TMW 2.1036	P. pent.	sourdough	heterofermentative	30
70	*Pediococcus (P.) pentosaceus* TMW 2.6	P. pent.	sake mash (DSM 20333)	heterofermentative	30
71	*Pediococcus (P.) pentosaceus* TMW 2.74	P. pent.	-	heterofermentative	30
72	*Pediococcus (P.) pentosaceus* TMW 2.8	P. pent.	beer yeast (DSM 20336)	heterofermentative	30
73	*Pediococcus (P.) pentosaceus* TMW 2.149	P. pent.	DSM 20280	heterofermentative	30
74	*Weissella (W.) cibaria* TMW 2.1333	W. cib.	sourdough	heterofermentative	30
75	*Weissella (W.) cibaria* TMW 2.1039	W. cib.	sourdough	heterofermentative	30
76	*Weissella (W.) confusa* TMW 2.1034	W. conf.	sourdough	heterofermentative	30

3.1.7 Primer

Oligonucleotides for screening, cloning and sequencing are listed in Table 7. They were purchased from Eurofins Genomics (Ebersberg, Germany). Specific primers were designed from single nucleotide sequences retrieved from NCBI database. Degenerated primers were designed from nucleotide sequence or protein sequence alignments of different LAB as described in section 3.2.1.1.

Table 7: Primer used in this work in alphabetical order. Primer sequences marked with (*) were taken from literature as indicated at respective site. Primer sequences marked with (**) were previously designed at Lehrstuhl für Technische Mikrobiologie using sequences of LAB (*L. plantarum, L. johnsonii, L. gasseri* und *L. acidophilus*) and yeast (*Burkholderia cenocepacia, Aspergillus flavus* und *Talaromyces stipitatus*) and have not been published yet. Recognition sites of restriction enzymes which were used for cloning are underlined.

Primer	**Primer sequence: 5´→ 3´**	**Use/ species or plasmid considered**
ABC_trans_f:	CCGAATCAGGCGTCACTA	Sequencing/ TMW 1.53 Δ*tcyB*
AFN2_lab_for* (Park et al. 2007)	GGNAAYGARATGGAYGG	Screening alpha- L- Arabinfuranosidase/ different strains
AFC_lab_rev* (Park et al. 2007)	CCANACRTTCCAYTCRTC	Screening alpha- L- Arabinfuranosidase/ different strains
cytB_for	TGGGAYGSVAAYGAAGT	Screening cytochrome B/ different strains
cytB_rev	AAGACMACTTCRCCNGC	Screening cytochrome B/ different strains
eryV	GACTCAAAACTTTATTACTTC	Sequencing/ *pME-1*
Fae_for**	CGNTTYGAYTTYRAYGG	Screening ferulic acid esterase/ different strains
Fae_rev**	ATNGGNWRYTGNGCNRTNG	Screening ferulic acid esterase/ different strains
glaldDH_for	GGNTTYGGNMGNATHGGNMGN	Screening glyceraldehyde- 3- phosphate dehydrogenase/ different strains
glaldDH_rev	CDATNGCYTTNGCNGCNCC	Screening glyceraldehyde- 3- phosphate dehydrogenase/ different strains
G_perox_F_1	GCGGGATCCTGAATGGTCGAGAAATTG	Cloning glutathione peroxidase (*Gpo*)/ TMW 1.53
G_perox_R_1	GCGGGATCCTGGAGAAGTTACTGGAGC	Cloning glutathione peroxidase (*Gpo*)/ TMW 1.53
GSHPerox_DNA_for	TRATYGTKAATACKGCHASBAA	Screening *Gpo*/ different strains
GSHPerox_DNA_rev	AAYTTYGTRAARTTCCAYTTGA	Screening *Gpo*/ different strains
Hyp_Gp_rev	CAGCAAATGATAGTGGGACAC	Sequencing/ *Gpo* mutant
msrA_for:	RTCYTGDAAHTGNCC	Screening peptide methionine sulfoxide reductase/ different strains
msrA_rev:	GSTNRRVCCDTTTGA	Screening peptide methionine sulfoxide reductase/ different strains
narH_for	CGYTGGGARGAYGAAGA	Screening nitrate reductase/ different strains
narH_rev	TATTCVGKTGADCCHGC	Screening nitrate reductase/ different strains
Ndh2_for	GGTTCYGGHTTYACBGG	Screening NAD(P)H dehydrogenase/ different strains
Ndh2_rev	CCGGAARAAYTCKTGCA	Screening NAD(P)H dehydrogenase/ different strains

Primer	**Primer sequence: 5´→ 3´**	**Use/ species or plasmid considered**
Npox_for	GTYGGKKCWKCWCAYGG	Screening NADH peroxidase/ different strains
Npox_rev	TCAATHCCRATRTADCCGS	Screening NADH peroxidase/ different strains
opep_for	ATHGAYGTNGTNGARAAYMA	Screening oligo(endo)peptidase F/ different strains
opep_rev	RTARTARAARTGNGGDATNCK	Screening oligo(endo)peptidase F/ different strains
pdc_for* (de las Rivas et al. 2009)	GANAAYGGNTGGGARTAYGA	Screening phenolic acid decarboxylase/ different strains
pdc_rev* (de las Rivas et al. 2009)	GGRTANGTNGCRTAYTTYT	Screening phenolic acid decarboxylase/ different strains
pep_C_for	TTYTGGGAYAARTTYGARAAR	Screening aminopeptidase C/ different strains
pep_C_rev	NACNACRTTNCCNARCATYTC	Screening aminopeptidase C/ different strains
pep_E_for2	GGNGGNCARTGGGCN	Screening aminopeptidase E/ different strains
pep_E_rev	NARNACRTCRTTNCCRAACCA	Screening aminopeptidase E/ different strains
pheS-pMTL_F2	GTTTTCCCAGTCACGACGTT	Sequencing/ *pMTL500e*
pheS-pMTL_R	CGGGGATCCTCTAGAGTCG	Sequencing/ *pMTL500e*
phgluc_for	YTNATHGAYGGNGGNAAYACN	Screening 6- phosphogluconate dehydrogenase/ different strains
phgluc_rev	AAYAARGGNACNGGNAARTGG	Screening 6- phosphogluconate dehydrogenase/ different strains
pMG36e_for	CGGAGGAATTTTGAAATGGC	Sequencing/ *pMG36e*
pMG36e_rev	AACTGTCTTGGCCGCTTCAA	Sequencing/ *pMG36e*
pMTL_for	GAGCCAACAGAACCAGAACC	Cloning/ *pMTL500e*
pMTL_rev	CCTGGCTTGGTAGTGATTGAG	Cloning/ *pMTL500e*
Pseu_for	GCCTGGTCTGACTGTGGT	Sequencing/ *Gpo* mutant
pyrP_na_r	CTATCGAAGCCGATACAACTG	Sequencing/ TMW 1.53Δ*tcyB*
Red_II_for	ATWKGATWTNACRWTTATTRRTKGNGG	Screening Ferredoxin- NADP reductase/ different strains
Red_II_rev	GGHTYRAWWGMBSCAHTYYC	Screening Ferredoxin- NADP reductase/ different strains
Rex_F_1	GCGGGATCCGCTTTATTATCGTTATT	Cloning transcriptional regulator *rex*/ TMW 1.53
Rex_R_1	GCGGGATCCTCGTCTTCATCACTCTT	Cloning transcriptional regulator *rex*/ TMW 1.53
SP6	GATTTAGGTGACACTATAG	Sequencing/ *pME-1*
spx_II_for	GAAGANATTATTTCWAMNCG	Screening transcriptional regulator *spx*/ different strains
spx_II_rev	CKRATTTCWTCTTCGTT	Screening transcriptional regulator *spx*/ different strains
T7	GTAATACGACTCACTATAGGGC	Sequencing/ *pME-1*

Primer	**Primer sequence: 5´→ 3´**	**Use/ species or plasmid considered**
tcyB_com_for4	ATCCATGGAAAAACTAAACGAAAGGACT GATT	Complementation into *pMTL500e*/ TMW 1.53Δ*tcyB*
tcyB_com_rev4	ATACCATGGAGTTCTCGTCCCCTTTCATA	Complementation into *pMTL500e*/ TMW 1.53Δ*tcyB*
tcyB_com_for5	ATTCTAGAAAAAACTAAACGAAAGGACT GATT	Complementation into *pMG36e*/ TMW 1.53Δ*tcyB*
tcyB_com_rev5	TATCTAGAAGTTCTCGTCCCCTTTCATA	Complementation into *pMG36e*/ TMW 1.53Δ*tcyB*
Th_red_F_1	GCGGGATCCGCTTGATCGTGGTGTTTA	Cloning thioredoxin reductase (*TrxR*)/ TMW 1.53
Th_red_R_1	GCGGGATCCACGAACATCACCAACAGC	Cloning thioredoxin reductase (*TrxR*)/ TMW 1.53
TrxR_AA_for3	TAYGGNGGNCARATGAAYAAYAC	Screening Thioredoxin reductase (*TrxR)*/ different strains
TrxR_AA_rev3	CRTCRCANACNGCRCARTA	Screening Thioredoxin reductase (*TrxR)*/ different strains
ytpP_for	TKRATRAAWTYTTCDACYTST	Screening Thioredoxin like protein (*ytpP*)/ different strains
ytpP_rev	YTGGTGYYCDGATTG	Screening Thioredoxin like protein (*ytpP*)/ different strains

3.1.8 Restriction enzymes

All restriction enzymes used in this work were provided by Thermo Scientific Fermentas (Waltham, MA, USA) and applied as recommended in the manual of the supplier. If not stated otherwise, Fast Digest (FD) enzymes were used.

3.1.9 Plasmids

3.1.9.1 pME-1

The former *pSP72* plasmid with an ampicillin resistance (β- lactamase) gene was extended with an erythromycin resistance (*ErmC*) gene of *Staphylococcus aureus* as visible in Figure 3. This non-replicating vector with a size of 3,971 bp was used to clone a fragment of the target gene into multiple cloning sites. After homologous recombination, the insertion into the chromosome takes place and the reading frame of the target gene is interrupted which results in loss of gene function.

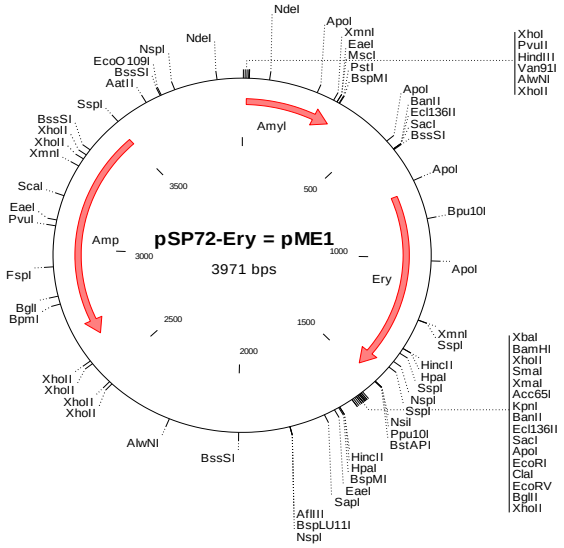


Figure 3: Vectormap of *pME- 1.*

3.1.9.2 pMTL500E

The shuttle vector *pMTL500E* was provided of the chair of Microbiology of the Technical University Munich and was originally isolated from *Bacillus subtilis.* As shown in Figure 4 the replicating vector carries erythromycin and ampicillin resistance genes, which are suited for selection in *E. coli* and *L. sanfranciscensis.* The vector consists of 6,380 bp and was used in this work mainly for complementation experiments and to control the transformation efficiency of electrocompetent *L. sanfranciscensis* cells.

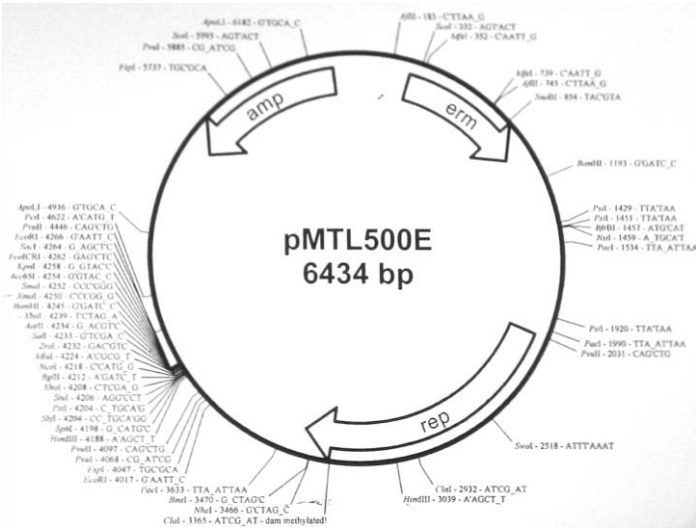


Figure 4: Vectormap of *pMTL500E*.

3.1.9.3 pmG36e

The vector *pmG36e* with its origin of replication *pWV01*, was constructed for the heterologous expression of genes in *Lactococcus lactis* subsp. *lactis*. It consists of approx. 3,700 bp. The kanamycin resistance cassette was replaced by an erythromycin resistance gene (designation from *pMG36* to *pmG36e*) of *Staphylococcus aureus* plasmid *pE194* for selection of plasmid carrying clones (van de Guchte et al. 1989) as visible in Figure 5. The promoter *p32* derives from *Streptococcus cremoris*. In this work, the vector was used for complementation experiments. It also comprises of a multiple cloning site flanked by gene expression signals including a termination signal that derive from *L. lactis* subsp. *cremoris* Wg2.

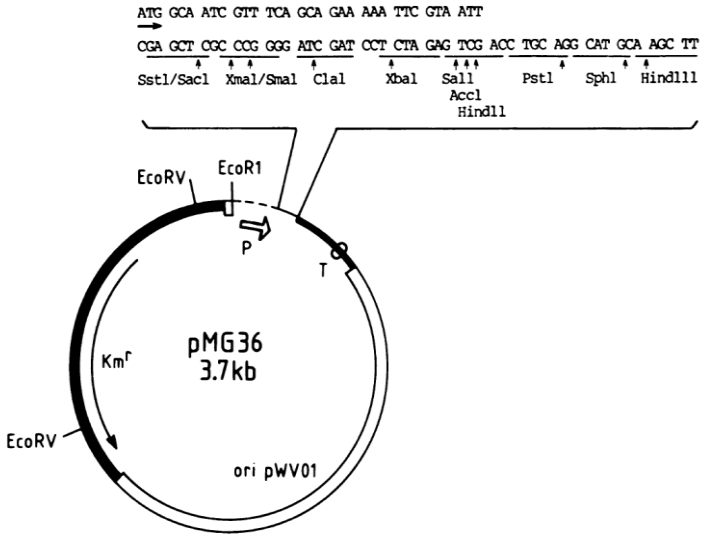


Figure 5:Vectormap of *pMG36e*.

3.2 Methods

3.2.1 Molecular biology methods

3.2.1.1 DNA isolation and primer design

For DNA isolation, the E.Z.N.A.® Bacterial DNA Kit (Norcross, USA) was used according to the protocol of the supplier with a few variations. The incubation times for lysozyme, proteinase and RNAse treatment were increased depending on the strain used for extraction; the elution buffer was prewarmed to 65 °C before applied onto the extraction columns to yield higher DNA concentrations. Resulting DNA was solved in TE buffer and the concentrations were measured with Nanodrop® ND-1000 (Thermo Scientific, USA). For PCR screening and for cloning purposes, the DNA concentrations were adjusted to 50 ng/ µL.

The primer design was carried out manually from annotated DNA and protein sequences taken from NCBI (http://www.ncbi.nlm.nih.gov/) of different *Lactobacillus* strains. Genes were searched according to the available annotations and information in the literature. The chosen genes were classified into the following main five groups: "typical redox", peptidase, dehydrogenase, electron transport chain (ETC) and "aroma". For primer design, multiple sequence alignments with DNA and protein sequences were performed with *ClustalW* (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Particularly conserved regions were used for manual primer design. Designed primers were checked with a primer analysis tool of Thermo Scientific to detect possible variations in melting temperatures, determination of CG contents (%), primer lengths, estimations of primer- dimer binding, molecular weights etc. (http://www.thermoscientificbio.com/webtools/multipleprimer/?redirect=true). Table 7 lists the degenerated PCR primers used in this work. In Appendix A1 to A 13 published DNA and protein sequences of the strains (see Table 6 for used abbreviations) with locations for manual primer design are displayed.

3.2.1.2 PCR performance

PCR amplification was performed by using a final volume of 25 µL containing 1.25 U of *Taq* DNA polymerase (MP Biomedicals, Solon, USA), 10 x appropriate buffer with 15 mM $MgCl_2$, deoxynucleoside triphosphate at a concentration of 0.2 mM, 0.4 µM of each primer and 100 ng of the DNA of the 76 tested strains. If necessary, primer binding was increased by the addition of 25 mM $MgCl_2$ stock solution with end concentrations of 2.5 mM or 3 mM $MgCl_2$. The thermal cycle involved 3- min activation of the polymerase at 94 °C before 30 cycles of dissociation (45- sec at 93 °C), annealing (30- sec, temperature variable) and elongation (1- min, 72 °C) followed. For determination of the annealing temperature, all used primers were tested in gradient- PCR and with DNA of different *Lactobacillus* strains to test the efficiency as positive controls.

For complementation experiment and sequencing-PCR, the proof-reading Phusion® DNA polymerase (New England Biolabs, USA) was used. PCR amplification with Phusion® DNA polymerase was performed by using a final volume of 50 µL containing 0.5 µL of Phusion® DNA polymerase (New England Biolabs, USA), 5 x appropriate HF buffer, deoxynucleoside triphosphate at a concentration of 0.2 mM, 0.4 µM of each primer and 100 ng of the DNA. The thermal cycle involved 3- min activation of the polymerase at 98 °C before 35 cycles of dissociation (10- sec at 98 °C), annealing (30- sec, temperature variable) and elongation (5- min, 72 °C) followed. The amplification of obtained PCR products of the expected size was confirmed by electrophoresis through a 1 % agarose gel (Biozym, Germany) in TBE or TAE buffer (Sambrook, 2001) followed by staining with dimidium bromide (Roth, Germany). The bands were visualized using UV light. Negative results were tested two times to exclude false negatives due to low DNA concentration and DNA degradation.

3.2.1.3 Analysis of PCR products using agarose gelectrophoresis

6 x Loading Dye

0.03 % Bromphenol blue

0.03 % Xylene Cyanol FF

60 % Glycerol

60 mM EDTA

10 mM Tris- HCl (ph 7.6)

PCR products were analyzed using 0.8 – 3 % agarose gels. The agarose was solved in TAE or TBE buffer and heated in the microwave. The gels were placed into gelelectrophoresis chambers filled with the appropriate buffer. The PCR products were mixed with Loading Dye whereas the 100 bp and 1000 bp markers of Fermentas (St. Leon- Rot, Germany) served as size standards. The average running time at standard conditions (120 V, 200 mA) was 60 – 120 minutes. After running, the gels were placed into dimidiume bromide (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) bath before they were analyzed using UV light for visualization. Appropriate bands were cut, purified with "QIAquick PCR purification Kit" (Qiagen GmbH, Hilden, Germany) and sent to the company GATC Biotech (Konstanz, Germany) for sequencing.

3.2.1.4 Construction of chemically competent E. coli and transformation

Calcium chloride solution

60 mM $CaCl_2$ x 2 H_2O

15 % Glycerin

10 mM PIPES

E. coli was precultured in 50 mL LB media at 37 °C. After inoculation in fresh media, the cells were incubated aerobically for 3 – 4 hours at 37 °C until the OD_{595nm} of 0.4 was reached. The cells were harvested through centrifugation for 5 minutes at 3,000*g* and 4 °C. The supernatant was removed and the cell pellet was carefully washed with 20 mL of 4 °C cold calcium chloride solution, before the volume was adjusted to 50 mL. After another centrifugation step at 3,000*g* the washing step was repeated once. Finally, the pellet was resuspended in 1250 µL calcium chloride solution at 4 °C and incubated on ice for 20 minutes. The suspension was aliquoted à 100 µL and shocked with liquid nitrogen for 5 minutes. The chemically competent *E. coli* cells were stored at – 80 °C for further use. For transformation, 100 µL of chemically competent *E. coli* cells were mixed with 5 µL of ligation mixture (or 1 ng of plasmid DNA) and placed on ice for 10 minutes. Afterwards, the bacteria were placed into a water bath with 42 °C for 2 minutes, the addition of LB or SOC media and incubation at 37 °C for 30 – 60 minutes recovers the cells. *E. coli* DH5α which were transformed with *pME-1* integration vectors were plated onto LB plates containing 100 µg/ mL of ampicillin for selection of positive clones.

3.2.1.5 Construction of electrocompetent L. sanfranciscensis TMW 1.53

The preparation of electrocompetent *L. sanfranciscensis* cells was performed as already described in the work Jänsch et al. (2007) with slight modifications. One colony of the strain was grown on mMRS (modified MRS, chapter 3.2.2.2) media added with 1 % (wt/vol) of glycine at 30°C in a water bath covered with aluminium foil to an optical density at 590 nm of 0.6. The cells were cooled on ice and centrifuged at 4°C (5,500*g*, 15 min), washed three times with 40 mL of 10 mM $MgCl_2$ solution, one time with glycerol (10%, vol/vol), one time with glycerol- sucrose solution (10%, vol/vol; 0.5 M). The resuspension of the cells were performed in glycerol- sucrose solution in which they were incubated on ice for 20 minutes, aliquoted à 80 - 100 µL, freezed in liquid nitrogen and stored at - 80°C for further use. All centrifugation steps were carried out at 4°C and all washing and storage solutions were cooled on ice before. The transformation efficiency of electrocompetent bacteria was checked using *pMTL500E* with a concentration of 50 – 100 ng/ µL.

3.2.1.6 Insertional inactivation of target genes by using single crossover integration

For insertional inactivation of known "redox genes", the following four genes were amplified based on the genome of *L. sanfranciscensis* TMW 1.1304: 1) thioredoxin reductase (*trxR*), 2) transcriptional regulator (*rex),* 3) glutathione peroxidase (*gpo, gpx*) and 4) Cystin transport permease (*tcyB*). The primers listed in Table 7 (Th_red_F_1/ R_1; Rex_F_1/ R_1; G_perox_F_1/ R_1; and Cys_F_1/ R_1) were used to obtain PCR products carrying BamHI restriction sites. Restriction endonuclease digestions and ligations with T4- DNA ligase were performed as indicated by the supplier (Fermentas, St. Leon- Rot, Germany). For insertional inactivation of the mentioned genes, the fragments were digested and ligated into plasmid *pME-1* resulting in the nonreplicating integration vectors *pME-*

1ΔtrxR, pME-1Δrex, pME-1Δgpo and *pME-1ΔtcyB.* The vectors were transformed in chemically competent *E. coli* DH5α for multiplication as described in section 3.2.1.4. The plasmid isolation was executed with the "Plasmid Mini Kit" (Omega, Norcross, USA). Positive clones onto agar plates were picked and streaked out a second time onto agar plates containing the appropriate antibiotic and checked with insert and vector primers in the colony- PCR as described in section 3.2.1.2. Selected PCR products with the proper size were purified with the "QIAquick Purification kit" (Qiagen, Hilden, Germany), sequenced by the company GATC Biotech (Konstanz, Germany) and checked with ChromasPro 1.7.5 software (http://technelysium.com.au/?page_id=13). Alignments were conducted with the online tool ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The isolated and purified plasmids of *E. coli* were used for transformation into *L. sanfranciscensis* TMW 1.53. Transformations of electrocompetent *L. sanfranciscensis* cells (as described in section 3.2.1.5) were carried out with a Bio- Rad gene pulser apparatus in 0.2 mm cuvettes (Bio- Rad Laboratories, Hercules, CA) with the following parameters for LAB: 1.2 kV, 25 µF and 1,000 Ω. After electroporation with the Gene pulser device of Bio- Rad (Bio- Rad Laboratories, USA), the cells were recovered with prewarmed mMRS medium and incubated in the water bath at 30°C for 5 hours prior to plating on mMRS plates with 10 µg/ mL erythromycin. After incubation in the water bath, erythromycin- resistant colonies were plated again onto mMRS (+10 µg/ mL erythromycin) plates and cultured in liquid media with 5 µg/ mL of erythromycin. For verification of the insertion of the plasmids into the chromosome, the chromosomal DNA of the erythromycin- resistant colonies was extracted as described in 3.2.1.1. PCR was carried out with primers targeting the regions upstream and downstream of the four mentioned genes and regions on the plasmid from *pME-1* (SP6, eryR) (see primer sequences in Table 7). The PCR products were sequenced at GATC Biotech (Konstanz, Germany) and evaluated as described above.

3.2.1.7 Complementation of L. sanfranciscensis ΔtcyB

For complementation of *L. sanfranciscensis ΔtcyB,* the *tcyB* gene including promoter were amplified using Phusion® High-Fidelity DNA polymerase (New England Biolabs, USA) and primers tcyB_com_for4/ rev4 (for vector *pMTL500e*) and tcyB_com_for5/ rev5 (for vector *pMG36e*). Further information regarding detailed primer sequence and restriction sites can be taken from Table 7. Corresponding PCR conditions including used chemicals and thermal profile were already described in chapter 3.2.1.2. Inserts were cloned into vectors *pMTL500e* and *pMG36e* and transformed into *E. coli* DH5α as described in 3.2.1.4. Positive clones were selected in using the appropriate antibiotics and checked with insert and vector primers. Positive clones were cultured in liquid LB (+ antibiotic) media before plasmid isolation followed. Sequencing primers PheS-pMTL-F2/R (for *pMTL500e*) and pMG36e_for/rev (for *pMG36e*) were used to amplify the desired PCR product. Sequencing was carried out by GATC Biotech (Konstanz, Germany) and obtained sequences were checked as mentioned above. The products of positive clones were transformed into electrocompetent *L. sanfranciscensisΔtcyB* using the parameters mentioned in the previous chapter 3.2.1.6.

3.2.2 Microbiological Methods

3.2.2.1 mMRS, Spicher and LB media

Table 8: mMRS for cultivation of *L. sanfranciscensis* WT and mutants, pH = 6.2.

component	concentration [% (w/v)]
Peptone from Casein	1
Yeast extract	0.5
Meat extract	0.5
K_2HPO_4 x $3H_2O$	0.4
KH_2PO_4	0.26
NH_4Cl	0.3
Cysteine- HCl	0.05
Tween 80	0.1
Maltose	1.5
Fructose	0.5

Table 9: Spicher for cultivation of other *Lactobacillae*, pH = 5.6.

component	concentration [% (w/v)]
Peptone from Casein	1
Yeast extract	0.7
Meat extract	0.2
Sodium gluconate	0.2
Sodium acetate trihydrate	0.5
Diammonium hydrogen citrate	0.5
KH_2PO_4	0.25
$MgSO_4$ x 7 H_2O	0.02
$MnSO_4$ x H_2O	0.01
$FeSO_4$ x 7 H_2O	0.005
Cysteine- HCl	0.05
Tween 80	0.1
Maltose	0.7
Fructose	0.7
Glucose	0.7

Table 10: LB media for cultivation of *E. coli*, pH = 7.

component	concentration [% (w/v)]
Peptone from Casein	1
Yeast extract	0.5
Sodium chloride	0.5

3.2.2.2 Media and growth conditions

All *Lactobacillus* spp. were cultivated in modified MRS (mMRS, see Table 8) after (Stolz et al. 1995) with addition of 5 g fructose per l, whereas vitamins were omitted. If no growth in mMRS media could be detected, Spicher media was used (see Table 9). The growth temperatures for the corresponding strains are shown in Table 6. For agar plates, 1.5 % agar was added. The components were dissolved in 800 mL H_2O_{dest}, the pH was adjusted by adding 4 M or 6 M HCl to 6.2. Sugars were dissolved separately in 200 mL H_2O_{dest}. For experiments with recombinant *L. sanfranciscensis* in liquid media, 5 µg/ mL of erythromycin was added to maintain the insertion into the chromosome. For selection of recombinants and cultivation on agar plates, 10 µg/ mL of erythromycin was used. 1 mL sterile filtrated (pore size Ø 0.2 µm) magnesium/ manganese 1000- fold stock solution ($MgSO_4$ * 7 H_2O 100 g/l, $MnSO_4$ * 4 H_2O 50 g/l) was added to the media after autoclaving. Liquid bacterial cultures were incubated in sterile plastic tubes. Agar plates were incubated anaerobically using Anaerocult (Merck, Darmstadt, Germany) systems in heat- sealed plastic bags or airtight incubation containers.
Escherichia coli strains were grown in LB medium. Components were dissolved in H_2O_{dest} and pH was adjusted to 7.0. For clone selection, the appropriate antibiotic was added to the desired end concentration. *E. coli* strains were aerobically cultivated at 37 °C. Liquid cultures were incubated in Erlenmeyer flasks or sterile plastic tubes at 180 rpm.

3.2.2.3 Measurement of optical density ($OD_{590\ nm}$)

The measurements of the optical density were conducted in TECAN readers (TECAN Deutschland GmbH, Germany) at 590 nm. For this purpose, 200 µL of the bacterial cultures were pipetted into microtiter plates in duplicate, whereas mMRS media without bacterial cells served as Blank which was subtracted from the raw data.

3.2.2.4 Growth experiments in different mMRS media

Overnight cultures of WT, Δ*gpo* and Δ*tcyB* were grown in mMRS (+5 µg/ mL erythromycin for mutants) for 24 hrs at 30 °C. After washing the cultures with Ringer solution, the OD was adjusted to 2.0. The different mMRS media according to the work of Jänsch et al. (2011) were used, with a few changes, visible in Table 11. Additionally, bacteria were grown in precultures without Mn^{2+} (mMRS7) and cysteine (mMRS8) and inoculated a second time in these media in regard to gain information about storage capacities in *L. sanfranciscensis* TMW 1.53. The different media used were inoculated at 1 % level with the bacterial cultures solved in Ringer solution. The growth was monitored measuring the OD_{590nm} described in section 3.2.2.4. The bacteria were plated for colony forming unit (cfu) determination.

Table 11: Modifications of mMRS media for growth experiments.

component	mMRS	mMRS2	mMRS3	mMRS4	mMRS5	mMRS6	mMRS7	mMRS8
manganese	+	-	-	-	+	+	-	+
cysteine	+	+	-	+	-	-	+	-
fructose	+	+	-	-	-	+	+	+

3.2.2.5 Fermentation experiments of WT, Δgpo, ΔtcyB and Δnox in mMRS

WT, Δ*gpo* and Δ*tcyB* mutants of *L. sanfranciscensis* TMW 1.53 were fermented in mMRS media at 30 °C for 24 hrs and pH, redox potential and oxygen partial pressure were measured automatically described below. The already characterized Δ*nox* mutant (Jänsch et al. 2011) was used as control. The sterilized fermenters were filled with 300 mL each of mMRS media. Before inoculation, the strains were grown over night in the same media (+ 5 µg/ mL of erythromycin for the mutants) washed one time in Ringer solution and the OD was set to 2.0. The inoculation dosage was 1%. Samples were taken every 2 hrs (T0, T2, T4, T6, T8, T10, T12, T24) for OD_{590nm} measurement, HPLC analytic (amino acids, sugars, acids, ethanol) and thiol group determination. For measurement of the optical density, 200 µL per sample were pipetted in duplicate into microtiter plates to analyze the OD at 590 nm in TECAN Spektraflour or Sunrise (TECAN Deutschland GmbH, Crailsheim, Germany). The measurement of mMRS media gave a blank which was subtracted from the generated raw data. The fermentation experiment was carried out with three independent replicates (n = 3).

Fermentations were carried out for 24 hours in fermenters (B. Braun Sartorius, Göttingen, Germany) at 30 °C. The parameters pH, oxidation- reduction potential (ORP) and oxygen partial pressure (pO_2) were measured using electrodes to record changes in these parameters automatically every 10 minutes. The pH was measured by using autoclavable pH electrodes (Mettler- Toledo, Greifensee, Switzerland). The calibration was accomplished with pH 7 and pH 4 calibration buffers. The pO_2 electrodes (Ingold, Greifensee, Switzerland) were calibrated employing N_2 and air.
ORP was measured by autoclavable redox electrodes Pt–Ag/AgCl (SCHOTT, Mainz, Germany). The integrity of the redox electrodes were checked using a standard redox solution of 240 mV (Hanna, Kehl am Rhein Germany). The redox potential E_h was calculated using the equation:

$$E_h = E_m + E_r$$

in which E_h refers to the redox potential of the normal hydrogen electrode (NHE), E_m to the recorded redox potential and E_r is the potential of the reference electrode at 30 °C (204 mV). As changes in the pH during the fermentation modify also the Eh, the redox potential was expressed at pH 7 according to (L. & Mirna 1959):

$$E_{h7} = E_h - [(7 - pH)\ \alpha]$$

in which α is the change per pH unit (E_h - pH; mV/ pH unit) which was determined before. In mMRS media the variation of E_h was 55.7 mV/pH unit at 30 °C.

3.2.2.6 Calculation of reduction, acidification and oxygen reduction rate

The E_{h7}, pH and pO_2 values of three different fermentations for each strain were processed mathematically for calculation of reduction rate V_r (dE_{h7}/dt, mV/h), acidification rate V_a (dpH/dt, pH unit/h) and oxygen reduction rate O_r (dpO_2/h, pO_2%/h) according to the method of Cachon et al. (2002) and Wick et al. (2003). This calculation was used for the determination of the maximum reduction rate V_{mr} (mv/h), the maximum acidification rate V_{ma} (pH unit/h), the maximum oxygen reduction rate O_{mr} (pO_2%/h) and corresponding time points: T_{mr} (h), T_{ma} (h) and T_{or} (h).

3.2.2.7 Analysis of organic acids, carbohydrates and free amino acids

For quantification of organic acids and ethanol, 400 µL of each sample was mixed with 4 µL 15 % perchloric acid and incubated over night at 4 °C to precipitate proteins. The formed precipitate was removed by centrifugation (30 min, 4 °C) and the supernatant was filtered (0.2 µm) and diluted 1:3 with water. The samples were stored at – 20 °C until analysis.

For quantification of the carbohydrates, 500 µL of each sample was mixed with 250 µL 10 % ZnSO4 and 250 µL NaOH (0.5 N). The samples were mixed carefully, incubated for 20 min at room temperature (RT) following a centrifugation step for 30 min to remove the formed protein precipitate. The supernatants were filtrated (0.2 µm), diluted with water 1:20 and frozen at – 20 °C until analysis.

For amino acid determination 1 mL of the sample volume were mixed with 50 µL undiluted perchloric acid and incubated over night at 4 °C for protein precipitation. The samples were centrifuged for 30 min at 4 °C, supernatants were filtered (0.2 µm) and diluted 1: 50 with 0.1 M HCl. The samples were frozen at – 20 °C until analysis.

Organic acids, ethanol and carbohydrates were analyzed by IEC dual analysis system ICS-5000 (Dionex, USA). Organic acids and ethanol were analyzed employing a ReproGel- H 9 lm (Dr. Maisch GmbH, Ammerbuch- Entringen, Germany) combined with a conductivity detector, suppressor and RI-101 detector (Shodex, München, Germany) for ethanol detection. The maintenance of the system at 30 °C was provided using 1 mM heptafluorobutyric acid (mobile phase) with a flow rate at 1 mL min^{-1} and 5 mM tetrabutylammonium hydroxide (anion suppression reagent). This method was developed modifying an application of Dionex (Document No. 031181-07). Carbohydrates were analyzed using a Carbopac PA20 column (Dionex, Sunnyvale, USA) combined with an electrochemical detector ICS-5000 (Dionex, Sunnyvale, USA), (Schwab et al. 2008). Water (A/B), 100 mM NaOH (C) and 1 M Sodium acetate were used as solvents applying the following gradient: 0 min, 37.5 % B and 25.5 % C; 24 min, 100 % C; 34 min, 100 % D; 44 min, 37.5 % B and 25.5 % C.

The concentrations of free amino acids were determined by reversed phase high performance liquid chromatography (RP- HPLC on an UltiMate 3000 HPLC system, Dionex, Germany) as already described by Schurr et al. (2013). A Gemini C18 column (Phenomenex, Aschaffenburg, Germany) was used for measurements. The mobile phases A (20 mM Na_2HPO_4, 20 mM NaH_2PO_4, 0.8 % tetrahydrofuran, pH 7.8) and B (30 % acetonitrile, 50 % methanol, 20 % HPLC- grade water) were employed with a flow rate of 0.8 mL/min (0 min, 0 % B; 16 min, 64 % B; 19 min, 100 % B; 22 min, 100 % B; 22.25 min, 0 % B). Amino acid separation and UV- mediated detection at 338 or 269 nm was performed with a Dionex UltiMate 3000 HPLC system (Dionex, Idstein, Germany), the column temperature was set to 40 °C. Precolumn amino acid derivatisation using o-phthalaldehyde-3-mercaptopropionic (OPA) acid and 9-fluorenylmethyl chloroformate (FMOC) was conducted in accordance to the work of Bartóak et al. (1994). The quantification of amino acids was performed using external standards and Chromeleon evaluation software version 6.80 (Dionex, Idstein, Germany).

3.2.2.8 Growth experiments of WT and ΔtcyB in mMRS without cysteine

Overnight cultures of WT and Δ*tcyB* were grown in mMRS media. After washing two times in Ringer solution, the OD_{590nm} was adjusted to 2.0. A stock solution of cystine was diluted to final concentrations of 0.01 M, 0.001 M and 0.0001 M in mMRS. Measurements were carried out in 96 well plates in a plate reader at 30 °C for 24 hrs monitoring the growth at OD_{590nm}. For that, 200 µL of mMRS without additional cysteine (but different concentrations of cystine) were pipetted into 96 well plates including 2 µL of bacterial cells. Three different biological replicates were prepared. Media without bacterial cells served as Blank. To compare the results of the 200 µL of the microtiter plate with a higher volume, 15 mL tubes were also inoculated. Significance was calculated using two-sided t- test.

3.2.2.9 Growth experiments of WT and ΔtcyB in chemical defined media (CDM)

The compounds listed in Table 12 were weighed, mixed and the pH was adjusted to 6.2. To maintain the biological activity of the added compounds freshly prepared media was sterile filtrated (0.2 µm) using Sarstedt filter. CDM without cysteine and cystine, with cysteine only and with cystine only were prepared to separate growth characteristics of the Δ*tcyB* mutant vs. WT.

Table 12: Composition of chemical defined minimal media for *L. sanfranciscensis, adjusted after* (Hebert et al. 2000). *****Chemical defined media was prepared either with cysteine, cystine or without cysteine and cystine.

Media component	concentration [% (w/v)]
Natriumacetate trihydrate	0.5
KH_2PO_4	0.3
K_2HPO_4	0.3
$MgSO_4 \cdot 7\ H_2O$	0.02
$MnSO_4 \cdot 4\ H_2O$	0.005
$FeSO_4 \cdot 7\ H_2O$	0.002
Tween 80	0.1
L- Alanine	0.01
L- Arginine	0.01
L- Asparagine	0.02
L- Cysteine*	0.02
Cystine*	0.01
Glycine	0.01
L- Histidine	0.01
L- Isoleucine	0.01
L- Leucine	0.01
L- Methionine	0.01
L- Phenylalanine	0.01
L- Proline	0.01
L- Serine	0.01
L- Threonine	0.01
L- Tryptophan	0.01
L- Tyrosine	0.01
L- Valine	0.01
Nicotinic acid	0.0001
Pantothenic acid	0.0001
Pyridoxal 5 phosphate	0.0002
Adenine	0001
Orot acid	0.001
Uracil	0.001
C- Quelle: Maltose	1

Overnight cultures of WT and Δ*tcyB* were grown in mMRS media. After washing two times in Ringer solution, the OD_{590nm} was adjusted to 2.0. The CDM without cysteine and cystine was used for growth measurements. Cysteine and cystine were manually added to CDM in three different concentrations (see Table 13). Three different biological replicates were prepared. Measurements were carried out in 96 well plates in a plate reader at 30 °C for 24 hrs monitoring the growth at OD_{590nm}. For that, 200 µL of CDM were pipetted into 96 well plates including 2 µL of bacterial cells. CDM without bacterial cells served as Blank.

Table 13: Concentrations of Cystine and Cysteine in CDM for growth determination.

Cystine concentrations (stock solution of 0.144 M solved in 0.5 M HCl)	**Cysteine (stock solution of 0.57 M solved in distilled water)**
72 µM	285 µM
7.2 µM	28.5 µM
0.72 µM	2.85 µM

3.2.2.10 Survival test after H_2O_2 and diamide treatment

Overnight cultures of WT, Δ*gpo* and Δ*tcyB* were grown for 24 hrs. Stock solutions of H_2O_2 and diamide were prepared with a concentration of 10 mM and sterile filtrated (0.2 µm). Stationary grown bacterial cultures were treated with H_2O_2 and diamide (final concentration ~ 1 mM), the control cultures were inoculated with distilled water instead of oxidants. The experiment was performed with three biological replicates. Samples were taken every 2 hours for CFU determination.

3.2.2.11 Shock experiments with diamide and H_2O_2

Cultures of WT, Δgpo and Δ*tcyB* were grown in mMRS media until the OD of 0.5. A stock solution of 50 mM of H_2O_2 (Merck, Darmstadt, Germany) and diamide (SIGMA- Aldrich, Steinheim, Germany) was prepared and sterile filtrated before addition to the exponentially grown cells of *L. sanfranciscensis* followed. The final concentration of both oxidants in the cultures was approx. 1.67 mM; distilled water was only added to the control cultures. After incubation at 30 °C for 1 hour, a sufficient volume of each sample was taken and frozen at – 20 °C for thiol group determination.

3.2.2.12 Extracellular and intracellular thiol group determination

KPM solution

0.1 M K_2HPO_4 containing 10 mM $MgSO_4$ * $7H_2O$, pH 6.5

The extracellular and intracellular thiol groups after H_2O_2 and diamide treatment were determined as already described in the work of Jänsch et al. (2007) with slight modifications. After centrifugation, the cells were washed two times with nitrogen- aerated KPM solution. The volume of 5 µL of a 10 µM of L- Cystine and 10 µL of 1 M D- Glucose solution was added to KPM cell suspensions. The tubes were incubated for 1 hour at 30 °C before the cells were centrifuged and the supernatant was transferred into new tubes and placed on ice for determination of extracellular thiol groups. The volume of 50 µL of 10 mM DTNB solution was added and incubation in the dark for 30 minutes at RT followed. The supernatants were pipetted into 96 well plates to measure the absorption at 412 nm in a plate reader FLUOstar Omega (BMG Labtech, Germany). KPM solution served as Blank.

For determination of intracellular thiol groups, the cell pellet was washed two times in KPM solution before it was solved in 200 µL distilled water, 4 µL 0.5 M EDTA, 10 µL 1 M Tris- HCl (ph=8.0), 20

µL 10 mM DTNB and 100 µL 10 % SDS solution. The mixture was incubated for 1 hour at 30 °C before the cell debris was removed by centrifugation. The absorption of the supernatant was measured again in micro titer plate at 412 nm in FLUOstar Omega. For quantification of thiol groups, several dilutions of L- Cysteine were prepared in KPM solution. The resulting data was exported into Excel and a standard curve was constructed for quantification purposes.

3.2.2.13 Influence of oxidants treatment on growth inhibition determined with plate assay

The resistance against different oxidizing compounds in *L. sanfranciscensis* TMW 1.53 (WT) and Δ*gpo* and Δ*tcyB* mutants was tested on agar plates with two different media types (mMRS, mMRS5= without cysteine and fructose) according to the work of Jänsch et al. (2011). To maintain the genomic insertion 5 - 10 µg/ mL of erythromycin was added to agar plates for the mutants. The plates were covered with 150 µL of overnight cultures of WT and Δ*gpo* and Δ*tcyB* mutants. After drying of the plates, sterile sensi- discs (BD Diagnostics, Heidelberg, Germany) were supplemented with H_2O_2 (final concentrations 5 mM and 2.5 mM), paraquat (final concentrations 250 mM and 500 mM) and diamide (final concentrations 1 M and 0.5 M) and placed in the middle of the agar plates. To compare the growth under normal conditions, separate plates with the addition of distilled water were used for each strain as positive controls. Plates were incubated for 48 hrs at 30 °C anaerobically for H_2O_2 and diamide, aerobically for paraquat. Due to the potentially light sensitivity of the oxidants, plates were covered with aluminium foil and incubated in the dark. The growth of inhibition was measured as the diameter of growth inhibition expressed in millimeter. Data was loaded into "*R*" (http://www.bioconductor.org) and p-values (≤ 5 %) were calculated with two sample t- test indicating significant effects.

3.2.2.14 Growth response in the presence of different reducing and oxidizing agents

Overnight cultures were grown in mMRS (+5 µg/ mL erythromycin for the mutants), washed two times in Ringer solution before the OD was adjusted to 2.0 as already described. As reducing agents cysteine, glutathione (GSH) and dithiothreitol (DTT); as oxidizing agents, H_2O_2 and diamide were used. After sterile filtration, stock solutions of 1 M were diluted to obtain the following concentrations: 100 mM, 40 mM, 20 mM, 10 mM, 5 mM and 1 mM. The growth was monitored as described in section 3.2.2.3. Growth was monitored for 24 hours at 30 °C with OD_{590nm} measurement every 60 minutes in TECAN Spektraflour or Sunrise (TECAN Deutschland GmbH, Crailsheim, Germany). Data was exported and analyzed in Excel and "*R*" (http://www.bioconductor.org) to visualize data and calculate appropriate p-values using two- sided ANOVA test. When ANOVA indicated a significant effect, pairwise comparisons were made with Tukey's HSD test.

3.2.2.15 Qualitative assessment of H_2O_2 accumulation

For qualitative assessment of H_2O_2 accumulation, plates with four different media compositions were used (1. mMRS, 2. mMRS – manganese, 3. mMRS – manganese – cysteine and 4. mMRS – cysteine). Corresponding mMRS media components and agar was weighed and the pH was adjusted to 6.2. The agar media mix was heated in the microwave until the agar solved completely. After sugars were added, the mixture was cooled down and Tetramethylbenzidine (TMB, 0.03 g solved in 100 µL DSMO) and Horseradish peroxidise (HRP, solved in water, stock solution of 1 mg/ mL) were added. Plates were dried and stored in the dark at 4 °C until further use.

WT Δ*gpo*, Δ*tcyB*, Δ*nox* and Δ*gshR* were precultured in mMRS (for mutants + 5 µg/ mL erythromycin) as described in 3.2.2.2. The mutants Δ*nox* and Δ*gshR* were used as internal controls. After OD measurement, the cultures were washed two times in Ringer solution before they were solved in Ringer. A volume of 10 µL of each strain were dropped onto two different plates and after drying, the plates were incubated anaerobically for 48 hours at 30 °C. For assessment of H_2O_2 accumulation, plates were exposed to oxygen for 10 minutes. Immediately after oxygen exposure and after 30 seconds, 3, 5 and 8 minutes, pictures were taken with a digital camera to evaluate the intensity of the color change at respective time points. Also color changes on the plates were scored by eye to confirm the images.

3.2.2.16 Quantification of H_2O_2

For quantification of H_2O_2, the method of Nakajima et al. (2003) was used with slight modifications. Cultures of WT, Δ*gpo*, Δ*tcyB* and Δ*nox* were grown anaerobically in mMRS and mMRS without manganese for 48 hrs. After incubation, stationary phase cells were washed two times in Ringer solution, resuspended in Ringer solution with addition of 0.5 % glucose before the OD was adjusted to 2. Each of the suspension was incubated for 3 hrs anaerobically and aerobically (220 rpm) at 30 °C and a sample of each suspension was drawn after 1 hour (T_1) and after three hours (T_3). After a centrifugation step (15.000 rpm for 3 minutes), 18 µL of the supernatant was used for quantification using the Quantitative Peroxide Assay Kit – Lipid (Thermo Scientific). The procedure was performed according to the protocol of the manufacturer. The determination was executed in duplicate for every strain. The absorption at 595 nm was measured in the FLUOstar Omega reader immediately after preparation. For quantification of the samples, H_2O_2 standards ranging from 8 - 1000 µM were prepared in methanol. Using linear regression, the absorption values of the samples were used for quantification.

3.2.2.17 In silico prediction of proteins involved in thiol disulfide redox metabolism of L. sanfranciscensis DSM20451^T

Protein sequences of genomic DNA, *pLS1* and *pLS2* (NC_015978.faa, NC_015979.faa, NC_015980.faa) of *L. sanfranciscensis* TMW 1.1304 were retrieved from FTP website

(ftp://ftp.ncbi.nih.gov/genomes/). Proteins were searched automatically for CXXC, CXXS and CXXT motifs. Using PSI- BLAST (Altschul et al. 1997) from NCBI, the selected proteins were checked for conserved domains. The parameters for BLAST were set to default. After this step, proteins were manually checked according to specific parameters as outlined in the work of Gopal et al. (2009). Modeling of 3D structure was carried out with 3D- JIGSAW Protein Comparative Modelling Server (http://www.bmm.icnet.uk/~3djigsaw/) published by Bates et al. (2002) and the SWISS- MODEL Server (http://swissmodel.expasy.org/workspace) as described in the work of Arnold et al. (2006). All parameters were set as default. Detailed information can be taken from the corresponding results section 4.17.

3.2.3 Transcriptional analysis

3.2.3.1 Microarray design and strain selection

Microarrays were spotted on the Agilent 8x15K platform (Agilent Technologies, Santa Clara, CA, USA) with a custom probe design comprising the published sequence of *L. sanfranciscensis* TMW 1.1304 (released by NCBI, Genbank accession no. NC_015978.1 for the chromosome, NC_015979.1 and NC_015980.1 for the two plasmids *pLS1* and *pLS2*).

3.2.3.2 Media and growth conditions

Table 14: MRS for cultivation of *L. sanfranciscensis* TMW 1.1304 and *C. humilis* TMW 3.191, pH = 6.2.

component	concentration [% (w/v)]
Peptone from Casein	1
Yeast extract	0.4
Meat extract	0.7
Sodium acetate trihydrate	0.5
Diammonium hydrogen citrate	0.2
Dipotassium phosphate	0.2
KH_2PO_4	0.25
$MgSO_4$ x 7 H_2O	0.02
$MnCl_2$ * 4 H_2O	0.005
Tween 80	0.1
Maltose (for *L. sanfranciscensis*)	2
Fructose (for *L. sanfranciscensis*)	0.7
Glucose (for *C. humilis*)	0.8

As the transcriptional response of *L. sanfranciscensis* TMW 1.1304 to different environmental stimuli was tested, the strain was grown in mMRS media with maltose as the only sugar to OD 0.6. For the co- cultivation with *C. humilis* (TMW 3.191), the yeast was grown in mMRS media with glucose to

OD 0.8. The five conditions applied are summarized in Table 15. The applied conditions were carried out at 30 °C for 2 hrs before RNA isolation started.

Table 15: Experimental conditions for microarray analysis.

conditions	application	(incubation) time
1) Anaerobic (control)	Standing culture	2 hrs
2) Aerobic	180 rpm	
3) + Fructose	media with fructose	
4) Co- cultivation with *C. humilis* (TMW 3.191)	1/100 ratio yeast/ bacteria	
5) Co- cultivation with *C. humilis* (TMW 3.191)	1/10 ratio yeast/ bacteria	

3.2.3.3 RNA isolation

Table 16: Extraction mixture for RNA isolation.

component	volume
Phenol/ Chloroform	500 µL
10 % SDS	30 µL
3 M Sodium acetate (pH 5.2)	30 µL
Glass beads (75 – 150 µm)	500 mg
TE buffer (or MRS)	400 µL

The cells were centrifuged at 30 °C at 15.000*g* for 15 minutes for harvesting. The bacterial and yeast cells were quenched with – 20 °C cold 60 % glycerol using 3 volumes of quenching solution and 1 volume of cells. After mixing, the samples were stored at – 20 °C. Following a centrifugation step at – 20 °C at 9.000 rpm for 10 minutes, cells were resuspended in 1mL TE buffer. The samples were placed into tubes containing extraction mixture and mixed manually for 2 minutes. Afterwards, the tubes were immediately frozen in liquid nitrogen and stored at – 80 °C overnight for RNA isolation. The cells were broken down using a FastPrep® machine (MP Biomedicals, Solon, USA) between the steps; the tubes were placed on ice. The cells were centrifuged at 20.800*g* for 1 minute at 4 °C to remove the cell debris. The supernatants were transferred into new tubes and an equal amount of chloroform was added which was cooled before use. The mixtures were centrifuged again for 1 minute. The supernatants were taken and mixed in a ratio of 1:1 with the capture buffer of the "High Pure RNA isolation Kit" (Roche, Basel, CH). The next steps were carried out as described in the protocol of the supplier. The *DnaseI* digestion was carried out for 90 minutes directly on the column to remove traces of DNA. For elution of the RNA, 50 µL of elution buffer was added onto the columns. The RNA was stored in two aliquots, one of 20 µL for further analysis and labeling and one back- up of 30 µL at – 80 °C. The RNA quantity and quality was checked using Nanodrop™ ND- 1000 (Peqlab Biotechnologie, Erlangen, Germany) and Spektrometer 3000 SmartSpec™ (Bio- Rad, Hercules, CA, USA).

3.2.3.4 Reverse transcription and degradation of mRNA

For annealing, 10 µg of each RNA sample was mixed with 1 µL of random Nonamers (Invitrogen, Carlsbad, CA, USA) and nuclease free water yielding a total volume of 11 µL. After mixing, an incubation step at 70 °C for 5 minutes followed before the samples were cooled at RT for 10 minutes. The mixture was spinned down and placed on ice. For reverse transcription 5 x buffer, 0.1 M DTT, dNTPs and Superscript® Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) were gently mixed and added to the mix of RNA and Nonamers. An incubation step for 3 hrs at 42 °C followed before the samples were cooled on ice. For degradation of mRNA, 2 µL of 2.5 M NaOH were added to the samples, mixed and shortly centrifuged. After incubation for 15 minutes at 37 °C, 10 µL of HEPES free acid were added before the solution was mixed and centrifuged again for further use.

3.2.3.5 Labeling with cyanine dyes and purification of amino allyl- modified cDNA

CyScribe GFX columns were placed into new collection tubes. 500 µL of capture buffer was added to each column and the unpurified cDNA products were transferred into each CyScribe column. After mixing with the pipette, samples were centrifuged at 13.800*g* for 30 sec. The liquid was discarded before 600 µL of 80 % ethanol was added to each column. A centrifugation step followed. This washing step was repeated two times before the columns were dried with an additional centrifugation step. The columns were placed into new collection tubes and 60 µL of freshly prepared 0.1 M sodium bicarbonate (ph 9.0) was pipetted directly onto the column. The tubes were incubated with elution buffer for 1 - 5 minutes at RT. The purified cDNA was collected with a centrifugation step at 13.800 *g* for 60 sec. This elution step was repeated.

All labeling and hybridization steps have to be carried out in the dark due to light sensitivity of the CyeDyes. For labeling amino allyl- modified cDNA were directly added into one aliquot of CyeDye NHS ester. Before usage, the CyeDye NHS esters were resuspended completely by pipetting. Each cDNA sample was allocated into equal volumes and mixed with each of the dye, resulting in two tubes per sample, one labeled with cyanine 5 (Cye5) and one with cyanine 3 (Cye3). An incubation step at RT for 60 – 90 minutes followed before 15 µL of 4M Hydroxylamine was added to each coupling reaction. It was mixed by stirring and incubated at RT for another 15 minutes. Before hybridization, the quality of the cDNA and labeling was checked again with Nanodrop™ ND- 1000. The hybridization for the 8x15K microarrays was executed according to the protocol 5.5 (2007) of the supplier (Agilent, Santa Clara, CA, USA). Cye3 and Cye5 labeled samples (each of approx. 300 ng) have to be mixed with 5 µL of 10 x Blocking Agent, 25 x Fragmentation buffer and water to a total volume of 20 µL for each array. Incubation at 60 °C for 30 minutes fragments residues of RNA. The addition of 26 µL of hybridization buffer and careful mixing results in the solution which will be placed onto the arrays. For that, tubes were placed on ice and loaded onto the eight arrays without introducing air bubbles. The entire array was placed in an array rack and in a hybridization oven for at

least 16 hours at 65 °C and 10 rpm. The washing of the array was performed using the supplied washing solutions of Agilent. The array was dried and scanned with Agilent microarray scanner. The parameters were set as follows: Hitchip Profile, channels R + G, resolution 5 µm, 16 bit TIFF, XDR 0.10, RPMT and GPMT. Laser lights of wavelengths at 532 nm for Cye3 and 635 nm for Cye5 dye were used to excite.

3.2.3.6 Quantification and microarray analysis

Fluorescent images were captured as multi- image- tagged image file format (TIFF) and quantification was carried out with the Imagene 7.5 software (Axon) (BioDiscovery, Marina del Rey, USA) at NIZO food research institute (Ede, Netherlands). The resulting files were opened as Excel file, columns "name" and "signal mean" were marked and sorted. Files were loaded into "*R*" (http://www.bioconductor.org) and mean group values were calculated. The group mean values were copied into txt file and later into Excel. All calculated mean values and standard deviation (STD) values were copied into one Excel file including all LSA numbers which derived from the genome of *L. sanfranciscensis* TMW 1.1304. With KAAS (KEGG Automatic Annotation Server; http://www.genome.jp/tools/kaas/) each gene of the genome of *L. sanfranciscensis* TMW 1.1304 was given a specific K number (Moriya et al. 2007). In Excel, the corresponding LSA numbers were assigned to the K numbers including all mean and STD values. Finally, depending on the tested condition, the ratios of the mean values were calculated (e.g. Fructose/ Anaerobic). For determination of colours for visualization with *iPath2* (Interactive Pathways Explorer 2; http://pathways.embl.de/), Excel function "IF" was used. Ratios above ≥ 2 (overexpression) were defined as "green", values ≤ 0.5 (underexpression) were defined as "red" and conditions between 0.5 and 2 were marked as "blue" for no differential expression. Loading the *txt* files with the values into *iPath2*, metabolic pathways for *L. sanfranciscensis* TMW 1.1304 were generated (Letunic et al. 2008).

3.2.4 RNA sequencing

3.2.4.1 Growth conditions and RNA isolation of L. sanfranciscensis (WT) and mutant (ΔtcyB)

WT and Δ*tcyB* were grown anaerobically at 30 °C in mMRS media as described in section 3.2.2.2. For the mutant, 5 µg/ mL of erythromycin was added to the media to maintain the stability of the genomic insertion. In the exponential phase (OD ~ 0.5), 68.4 mM of diamide [1,1′- Azobis(*N*,*N*-dimethylformamide), Sigma- Aldrich, St. Louis, USA] was added to 40 mL cultures (final concentration of 1.7 mM). The control cultures were treated with distilled water instead of diamide. After incubation at 30 °C for 35 minutes without shaking, the cultures were shocked with - 20 °C cold methanol and centrifuged at 6000 rpm. The resulting pellet was solved in TE buffer before the cells were disrupted with Silica beads and the isolation with the "RNA Midi Kit" (Qiagen, Hilden,

Germany) started following the recommendations of the supplier. Two biological replicates were prepared on two individual days. The RNA was eluted from the columns with RNAse free water and the quantity was determined with Nanodrop® ND- 1000 (Peqlab Biotechnologie, Erlangen, Germany). For quality check, the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) was used. Remaining DNA was removed with the "TURBO DNA- free Kit™" (Ambion, Santa Clara, CA, USA) according to the protocol of the supplier. The RNA precipitation was carried out with ice- cold 3 M sodium acetate and abs. ethanol. After this step, the quality and quantity of the samples were checked again as already mentioned above. Subsequently, RNA samples were mixed with the reagent RNA stable® (Biomatrica, San Diego, CA, USA) and dried at room temperature under the flow hood. The dried RNA samples were sent to BGI Hongkong for library construction and RNA sequencing using Illumina HiSeqTM2000 technology. The samples have to fulfill the following requirements for RNA sequencing analysis at BGI (Hongkong): 1) sample quantity ≥ 10 µg; 2) sample concentration ≥ 65 ng/ µL; sample purity $OD_{260/280}$ = 1.8 – 2.0 and $OD_{260/230}$ ≥ 1.8; RNA 23S/ 16S ≥ 1.0 and RIN ≥ 6.0; treated with DNAse.

3.2.4.2 Data analysis

The data analysis was performed according to the protocol of Trapnell et al. (2012). The mapping of the generated paired- end FASTAQ files was carried out using *Bowtie* (Langmead et al. 2009) onto the published genome of *L. sanfranciscensis* TMW 1.1304 and the plasmids *pLS1* and *pLS2*. The resulting *sam* files were sorted and filtered with *samtools*. Generated bam files were provided as input to *cufflinks* which produced assembled transcript fragments with FPKM (fragments per kilobase of exon per million reads mapped) values. The programme *cuffmerge* was used to merge these data files into a single transcript with taking the reference transcriptome annotation data. For quantification of any differential expression, *cuffdiff* was applied in using standard settings. The quantified data were imported into "*R"* (http://www.bioconductor.org) and further processed with the *cummeRbund* package to generate expression plots. With specific commands differentially expressed transcripts and differentially spliced and regulated genes can be accessed. In this work the package was mainly used to identify genes which show significantly differentially expressed isoforms depending on the different treatments and to generate expression plots. The existing *bam* files of WT and mutant were changed into *tmp* files and loaded together with the FASTA files of the genome and the two plasmids of *L. sanfranciscensis* TMW 1.1304 into *DNAPlotter* and *Integrative Genomic Viewer (IGV)* (Carver et al. 2009; Thorvaldsdóttir et al. 2013).

4 RESULTS

4.1 Construction of knock-out mutants

The construction of knock-out mutants for thioredoxin reductase (*trxR*), redox- sensing repressor (*rex*), glutathione peroxidase (*gpo, gpx*) and cystine transport permease (*tcyB*) was the first objective. The transformation failed for the nonreplicating integration vectors *pME-1ΔtrxR* and *pME-1Δrex*. Using different plasmid concentrations of *pME-1Δgpo* and *pME-1ΔtcyB*, two clones for Δ*gpo* and Δ*tcyB* could be gained with 50 – 100 ng/ µL of the corresponding isolated plasmid.

For verification of insertion of *pME-1ΔtcyB* and *pME-1Δgpo* at appropriate site in the genome of *L. sanfranciscensis*, vector primers (SP6, eryR) were combined with primers flanking the target region in the chromosome (Gene_for/ Gene_rev) as visible from Figure 6. As there are two integration possibilities, specific PCR in the two clones (Δ*tcyB* and Δ*gpo*) revealed that integration I had taken place.

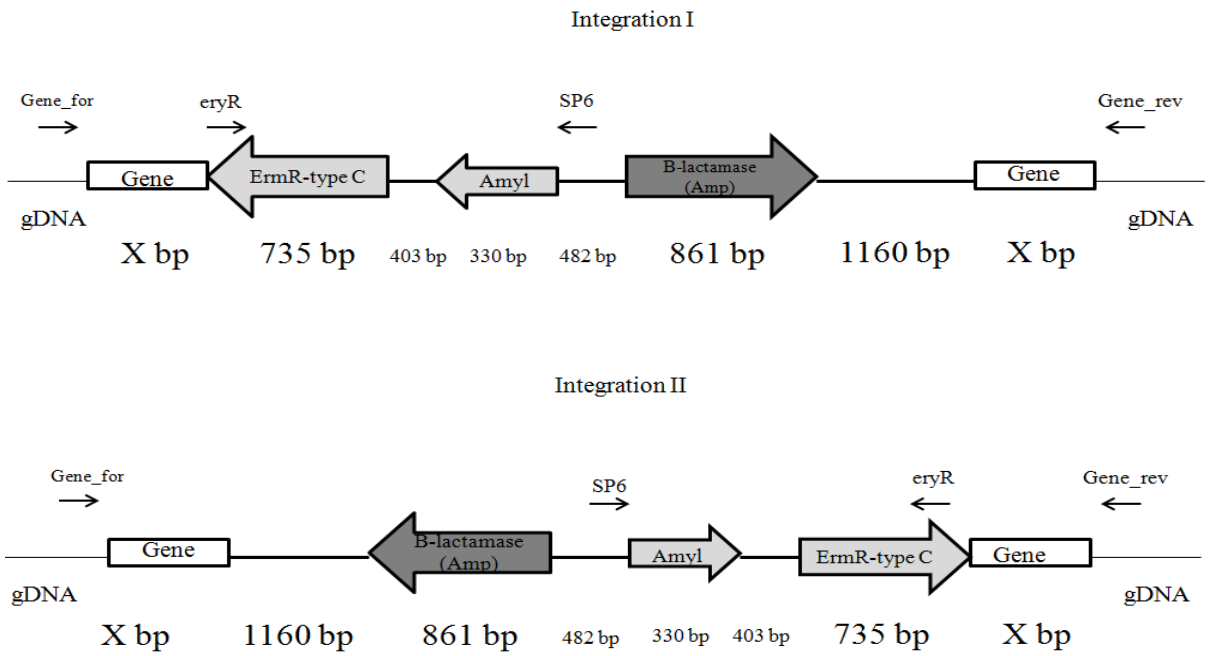


Figure 6: Schematic representation of both integration possibilities (I, II) of the non- replicating plasmid *pME-1* (including β- lactamase, amylase and erythromycin resistance genes) into genomic DNA (gDNA) of *L. sanfranciscensis* TMW 1.53 at respective site. Visible are the flanking regions (gDNA) with Gene_for and Gene_rev primers and the vector *pME-1* marked in bold. The combination of vector primers (SP6, eryR) and genomic DNA primers (Gene_for, Gene_rev) were used for verification of the genomic insertion. For integration I Gene_for/ SP6 and Gene_rev/ eryR result in PCR products; for integration II Gene_for/ eryR and Gene_rev/ SP6 were applied.

The PCR with ABC_for/ SP6 (Δ*tcyB*) and Pseu_for/ SP6 (Δ*gpo*) resulted in products of ~ 2100 and ~ 2300 bp whereas pyrP_rev/ eryR (Δ*tcyB*) and Hyp_Gp_rev/ eryR (Δ*gpo*) gave products with ~ 4500 and 4900 bp as visible from Figure 7. Chromosomal DNA of the WT (marked as 1.53 in Figure 7) served as negative control to eliminate products resulting from unspecific binding of the flanking primers in the chromosome; further for testing the specificity of the vector primers eryR and SP6. PCR

products resulting from Gene_for/ SP6 of Δ*tcyB* and Δ*gpo* were sequenced to verify the disruption of the genes. The sequences can be retrieved from Appendices A 14 and A 15.

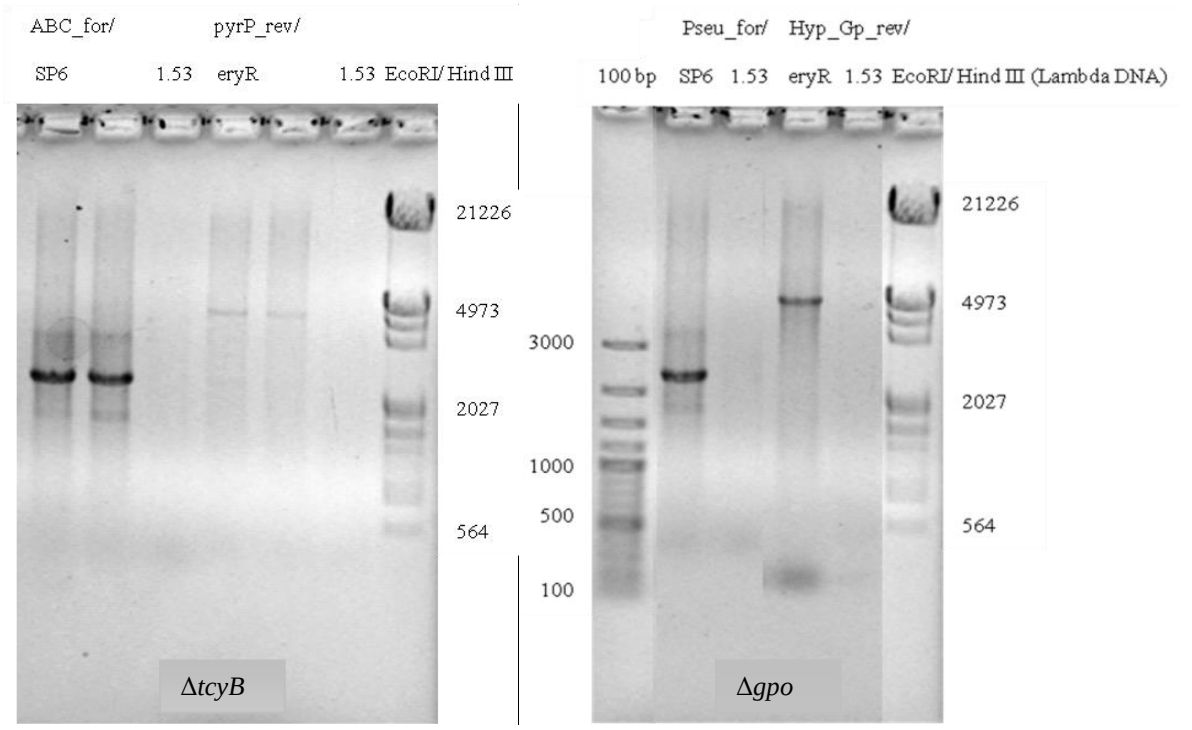


Figure 7: Agarose gel pictures after screening- PCR with border and vector primers. In the left picture, primers with the combination of ABC_for/SP6 and pyrP_rev/eryR yielded positive PCR bands for the Δ*tcyB* mutant. On the right, PCR bands resulting from primer combinations Pseu_for/ SP6 and Hyp_Gp_rev/eryR are visible for the Δ*gpo* mutant. Chromosomal DNA of the WT (TMW 1.53) served as "negative control". For estimation of the size of the PCR products, 100 bp marker and marker with Lambda DNA cut with *EcoRI* and *HindIII* were used.

4.2 Complementation of *L. sanfranciscensis* Δ*tcyB*

The complementation of *L. sanfranciscensis*Δ*tcyB* was conducted to reveal, if possible effects which are further described in this work, occurred due to the *tcyB* gene deletion or other mechanisms (e.g. introduction of the erythromycin resistance gene). Using the two vectors *pMTL500e* and *pMG36e*, positive clones could be obtained using both plasmids. Detailed analysis of the clones after transformation into *E. coli* DH5α indicated that the DNA quality of the clone transformed with *pMTL500e* was not good enough for sequencing. After sequence analysis, three positive clones for *pMG36e* resulted in a shorter *tcyB* insert where 24 bases were missing. The further transformation of these three plasmids into *L. sanfranciscensis* Δ*tcyB* yielded no positive clones.

4.3 Growth experiments in different mMRS media

Growth was tested in different mMRS media as already outlined in the corresponding methods section. Different growth behavior of the WT vs. mutants shall give first information concerning specific growth responses in different media and under aerobic and anaerobic growth conditions.

Taking the OD values of the strains, the growth rate µ (dOD_{590}/ dt) was determined for each media type. The µ values are visible in Table 17 whereas maximum and minimum were specifically marked. The values of the WT were higher during aerobic than anaerobic conditions. The same observation could be found for the two mutants Δ*gpo* and Δ*tcyB* except for mMRS2 (without Mn^{2+}).

Table 17: Summarized table of the maximum and minimum µ (dOD_{590}/ dt) values of WT, Δ*gpo* and Δ*tcyB* incubated anaerobically and aerobically in different media (mMRS1 - mMRS8). The highest values are underlined and marked in bold; the lowest values are marked in italic.

µ (dOD_{590}/ dt)	**mMRS1**	**mMRS2**	**mMRS3**	**mMRS4**	**mMRS5**	**mMRS6**	**mMRS7**	**mMRS8**	**MAX**	**MIN**
WT anaerobic	**0,125**	0,087	0,043	*0,037*	0,080	0,112	0,049	0,123	0,125	0,037
WT aerobic	0,146	0,107	0,111	0,087	0,138	0,122	*0,072*	**0,151**	0,151	0,072
Δ*gpo* anaerobic	0,085	0,068	0,006	*0,004*	0,023	**0,086**	0,019	0,057	0,086	0,004
Δ*gpo* aerobic	**0,097**	0,050	*0,040*	0,041	0,074	0,087	*0,040*	0,070	0,097	0,040
Δ*tcyB* anaerobic	**0,088**	0,053	0,008	0,006	0,009	0,027	0,008	*0,005*	0,088	0,005
Δ*tcyB* aerobic	**0,096**	0,050	0,096	0,061	0,019	0,013	0,039	*0,009*	0,096	0,009

The growth rates of the WT were overall higher compared to Δ*gpo* and Δ*tcyB*. WT reached the highest growth rate values in mMRS1 media under anaerobic conditions and in mMRS8 under aerobic conditions (preculture without cysteine). The lowest growth rates for the WT could be observed in mMRS4 during anaerobic conditions (without Mn^{2+} and fructose) and mMRS7 (preculture without Mn^{2+}) during aerobiosis. The Δ*gpo* mutant reached the highest values in mMRS6 (without cysteine) during anaerobic conditions, in mMRS1 during aerobic conditions. The lowest growth rate during anaerobiosis was determined in mMRS4 (without Mn^{2+} and fructose) as seen for the WT. During aerobiosis the lowest growth could be observed in mMRS3 (without Mn^{2+}, cysteine and fructose) and mMRS7 (without Mn^{2+}). The Δ*tcyB* mutant showed the highest growth rates during aerobic and anaerobic conditions in mMRS1. The lowest values in the presence and absence of oxygen in mMRS8 (preculture without cysteine).

4.4 Qualitative determination of H_2O_2 accumulation

For first screening purposes, the accumulation of H_2O_2 was monitored over 8 minutes using different agar plates (mMRS, mMRS – Mn^{2+}, mMRS – cysteine – Mn^{2+}, mMRS – cysteine). The generated images taken after 30 seconds, 3 and 8 minutes after oxygen exposure are visible in Figure 8. Corresponding color changes were scored by eye; the results are summarized in Table 18. Additionally to the Δ*gpo* and Δ*tcyB* mutants, Δ*nox* and Δ*gshR* were used as internal control.

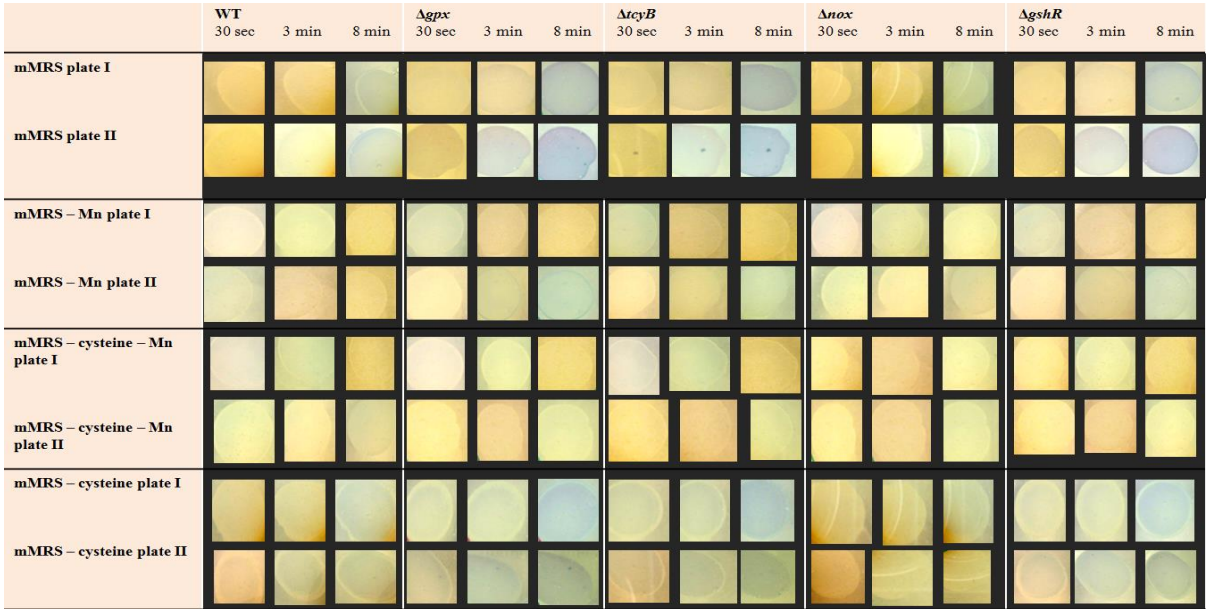


Figure 8: Pictures of the H_2O_2 plate assay of WT, Δ*gpo*, Δ*tcyB*, Δ*nox* and Δ*gshR* in four different media (mMRS, mMRS – manganese, mMRS – cysteine – manganese and mMRS – cysteine). Visible are the color changes of stationary grown cultures shortly after oxygen exposition, pictures were taken at time points: 30 seconds, 3 and 8 minutes. The experiment was conducted in duplicate on two different days, which is indicated as plate I and plate II.

The accumulation of H_2O_2 led to a color changes from white to blue. After 8 minutes the blue color for Δ*gpo*, Δ*tcyB* and Δ*gshR* was more pronounced than the blue color for the WT in mMRS. The Δ*nox* mutant showed no color change in mMRS media. Looking at the mMRS – Mn^{2+} and mMRS – cysteine – Mn^{2+} plates, the effect of the distinct color change was less visible. Only the mMRS – Mn^{2+} plates II with Δ*gpo*, Δ*tcyB* and Δ*gshR* showed a slightly blue stain after 8 minutes which was more pronounced than for the WT. The Δ*nox* mutant showed again no color change when Mn^{2+} and/ or cysteine were absent.

The plates with mMRS – cysteine showed a similar color change like mMRS plates. The blue stain for Δ*gpo*, Δ*tcyB* and Δ*gshR* was more pronounced compared to the WT. No color changes could be observed for Δ*nox*. Especially the margins of the bacteria appeared brighter for all tested strains.

Table 18: H_2O_2 accumulation on mMRS, mMRS – cysteine+ manganese, mMRS – manganese and mMRS– manganese– cysteine plates with TMB and HRP of the WT, Δ*gshR*, Δ*gpx*, Δ*tcyB* and Δ*nox*. Color changes after distinct time spans were scored by eye whereas 0 indicates no color change, 1 a slightly blue staining and 2 a clear blue staining. The experiment was conducted in duplicate; this table represents the results after single determination.

media	time	strains				
		WT	**Δ*gshR***	**Δ*gpo***	**Δ*tcyB***	**Δ*nox***
mMRS	0	0	0	1	1	0
	30 sec	0	0	1	1	0
	3 min	1	1	2	2	0
	5 min	1	1	2	2	0
	8 min	2	2	2	2	0
		WT	**Δ*gshR***	**Δ*gpo***	**Δ*tcyB***	**Δ*nox***
mMRS - cysteine	0	0	0	0	0	0
	30 sec	0	0	1	1	0
	3 min	1	1	1	1	0
	5 min	1	2	2	2	0
	8 min	1	2	2	2	0
		WT	**Δ*gshR***	**Δ*gpo***	**Δ*tcyB***	**Δ*nox***
mMRS - Mn	0	0	0	0	0	0
	30 sec	0	0	0	0	0
	3 min	0	0	0	0	0
	5 min	0	0	0	0	0
	8 min	0	1	1	1	0
		WT	**Δ*gshR***	**Δ*gpo***	**Δ*tcyB***	**Δ*nox***
mMRS - cysteine - Mn	0	0	0	0	0	0
	30 sec	0	0	0	0	0
	3 min	0	0	0	0	0
	5 min	0	0	0	0	0
	8 min	0	0	0	0	0

The images were additionally scored by eye (Table 18). The results mainly resembled the color differences showed above. The Δ*gpo* and Δ*tcyB* mutants showed faster and more pronounced color change in mMRS and mMRS – cysteine compared to the WT. The results for Δ*gshR* were comparable with Δ*gpo* and Δ*tcyB.* No color changes could be observed for Δ*nox* independent of the media examined. Using media without Mn^{2+}, color changes could not be detected for all strains.

4.5 Quantification of H_2O_2 in mMRS and mMRS without Mn^{2+}

The plating assay revealed that *L. sanfranciscensis* accumulates H_2O_2 in the presence of oxygen and depending on the incubation media.

The amount of accumulated H_2O_2 in anaerobic and aerobic cultures in mMRS and mMRS without Mn^{2+} can be retrieved from Figure 9 and Figure 10. The Δ*nox* mutant was used to compare the obtained results.

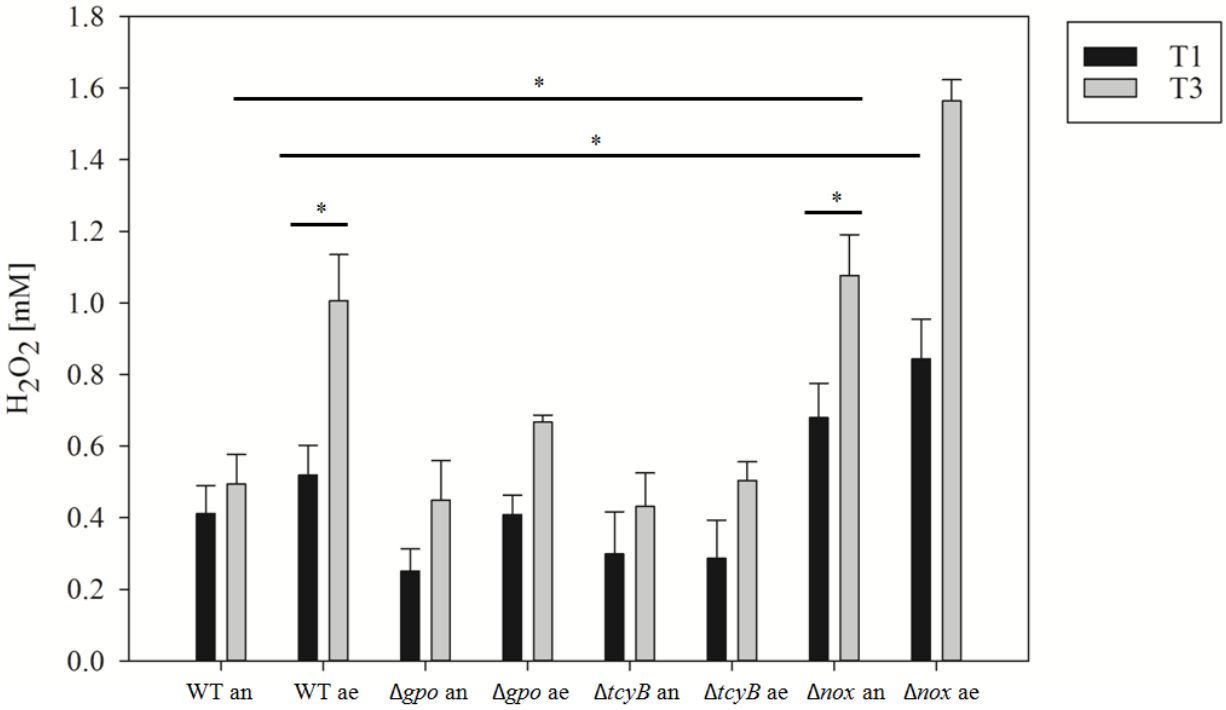


Figure 9: H_2O_2 quantification [mM] of WT, Δ*gpo*, Δ*tcyB* and Δ*nox* grown in mMRS. At the bottom the bar charts are labeled with the corresponding name (WT, Δ*tcyB*, Δ*gpo* and Δ*nox*) and anaerobic (an) or aerobic (ae), depending on the method of incubation. Shown are the mean values of triplicate measurements including standard deviations. Samples were taken and quantified after 1 hour (T1) and three hours (T3). *$p \leq 0.05$

In mMRS the WT accumulated significantly more H_2O_2 after 3 hours during aerobic incubation (1 mM vs. 0.5 mM). This effect could not be seen for the Δ*gpo* and Δ*tcyB* mutants. The amount of H_2O_2 during aerobic conditions was slightly higher but not significant compared to anaerobic growth conditions.

The Δ*nox* mutant accumulated more H_2O_2 during anaerobic and aerobic conditions than the WT. Already after 1 hour over 0.6 mM of H_2O_2 and after 3 hours of aerobic incubation over ~ 1.6 mM of H_2O_2 could be quantified.

The results concerning the amount of accumulated H_2O_2 in mMRS – Mn^{2+} (Figure 10) differed from the ones above.

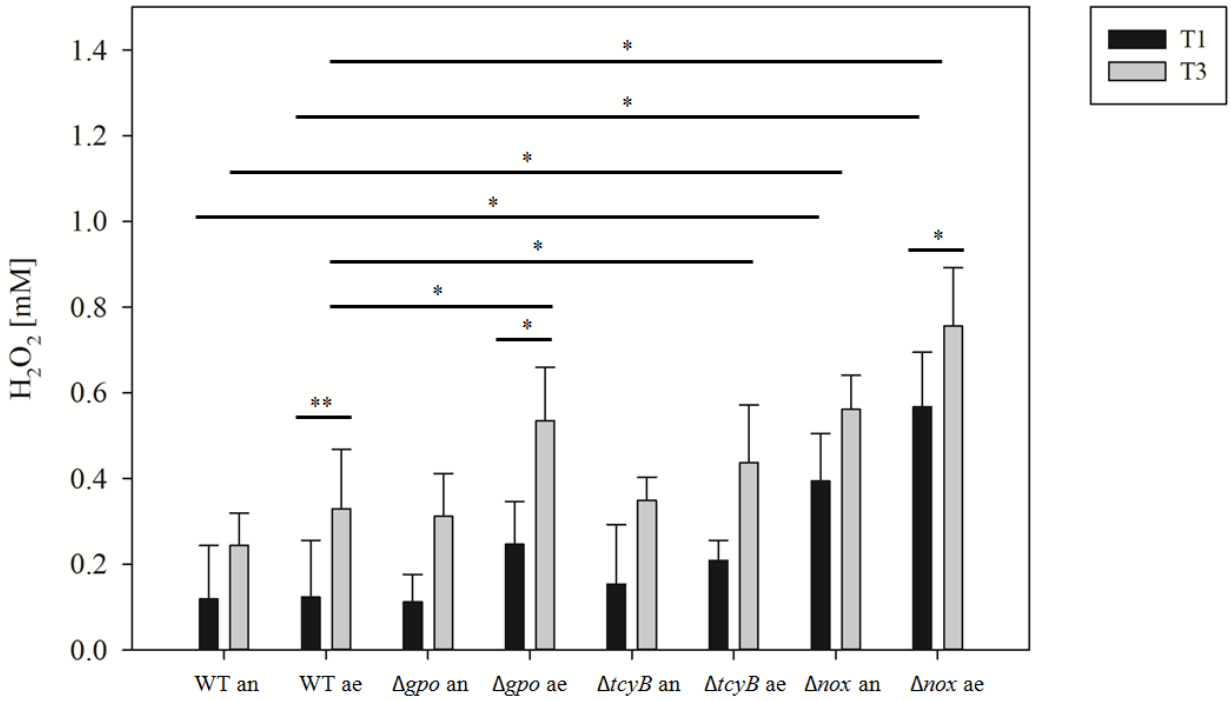


Figure 10: H_2O_2 quantification [mM] of WT, Δ*tcyB,* Δ*gpo* and Δ*nox* grown in mMRS <u>without Mn^{2+}</u>. At the bottom the bar charts are labeled with the corresponding name (WT, Δ*tcyB,* Δ*gpo* and Δ*nox*) and anaerobic (an) or aerobic (ae), depending on the method of incubation. Shown are the mean values of triplicate measurements including standard deviations. Samples were taken and quantified after 1 hour (T1) and three hours (T3). *p ≤ 0.05, ** p ≤ 0.01

Without additional Mn^{2+} in mMRS, overall less H_2O_2 was detected for all strains. The WT produced significantly more H_2O_2 after 3 hours of aerobic incubation. The same observation could be seen for the Δ*gpo* and Δ*nox* mutant. The Δ*gpo* mutant produced aerobically more H_2O_2 compared to the WT. The amount of produced H_2O_2 in the Δ*tcyB* mutant was also significantly higher compared to the WT (0.33 ± 0.14 vs. 0.44 ± 0.14) but only in the presence of additional oxygen.

The Δ*nox* mutant showed again a higher H_2O_2 accumulation independent of the presence/ absence of oxygen or the incubation time. After 3 hours of anaerobic incubation, approx. 0.6 mM, in the presence of oxygen ~ 0.8 mM of H_2O_2 could be measured. In comparison, the WT reached anaerobically approx. 0.2 mM and aerobically 0.3 - 0.4 mM of H_2O_2.

4.6 Growth response of WT and Δ*tcyB* to different cystine concentrations

To test the specificity and impact of the deleted cystine transporter (*tcyB*) in *L. sanfranciscensis*, the growth of Δ*tcyB* mutant and WT was monitored in mMRS without cysteine but supplemented with cystine. The OD_{590} values at respective time points can be taken from Table 19.

The WT reached after 10, 20 and 40 hours significant higher OD values compared to the Δ*tcyB* mutant. As growth control served normal mMRS in which the WT reached an OD of 1 and Δ*tcyB* an OD of 0.8. Growth was monitored also in higher volumes which is exemplary visible in Figure 11. Growth of WT and Δ*tcyB* at 0.1 mM cystine (left) and 1 mM cystine (right) was visible after 24 hours of incubation. Clearly visible was the normal growth behavior for the WT whereas Δ*tcyB* was not able to grow.

Table 19: Summarized table of the OD values of WT and Δ*tcyB* at specific time points (10 h, 20 h and 40 h). Visible are the mean OD 590 values including standard deviations of growth in mMRS without cysteine and different concentrations of added cystine (0.01 M, 0.001 M and 0.0001 M). The experiment was conducted in triplicate. *p ≤ 0.01.

Strain (concentration of cystine in M)	10 h	20 h	40 h
WT (0.01 M cystine)	0.11 ± 0.006	0.24 ± 0.014	0.38 ± 0.023
WT (0.001 M cystine)	0.13 ± 0.012	0.27 ± 0.021	0.44 ± 0.037
WT (0.0001 M cystine)	0.14 ± 0.012	0.30 ± 0.006	0.49 ± 0.013
WT mMRS	0.25 ± 0.026	0.99 ± 0.082	1.13 ± 0.080
Δ*tcyB* (0.01 M cystine)	0.01** ± 0.003	0.05** ± 0.009	0.14** ± 0.007
Δ*tcyB* (0.001 M cystine)	0.02** ± 0.012	0.07** ± 0.019	0.14** ± 0.020
Δ*tcyB* (0.0001 M cystine)	0.01** ± 0.003	0.06** ± 0.010	0.13** ± 0.009
Δ*tcyB* mMRS	0.12** ± 0.020	0.55** ± 0.039	0.78** ± 0.076

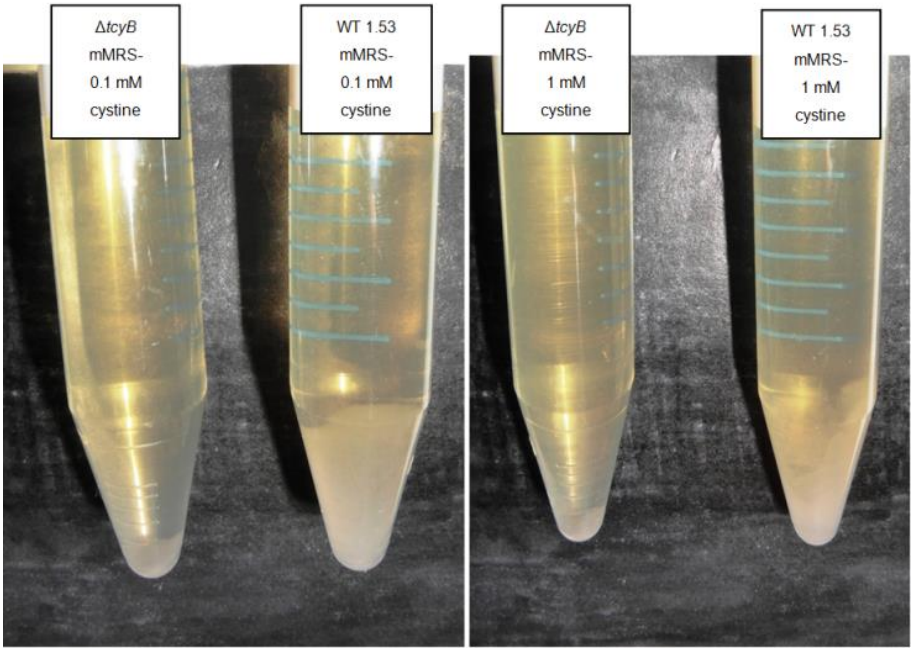


Figure 11: Growth of WT and Δ*tcyB* in mMRS without cysteine but with 0.1 mM cystine (left picture) and 1 mM cystine (right picture). Pictures were taken after 24 hours of incubation.

4.7 Growth tests in chemically defined media

To confirm the results gained from growth in mMRS without cysteine, WT and Δ*tcyB* were grown in CDM with additional cystine (visible in Figure 12) and cysteine (visible in Figure 13).

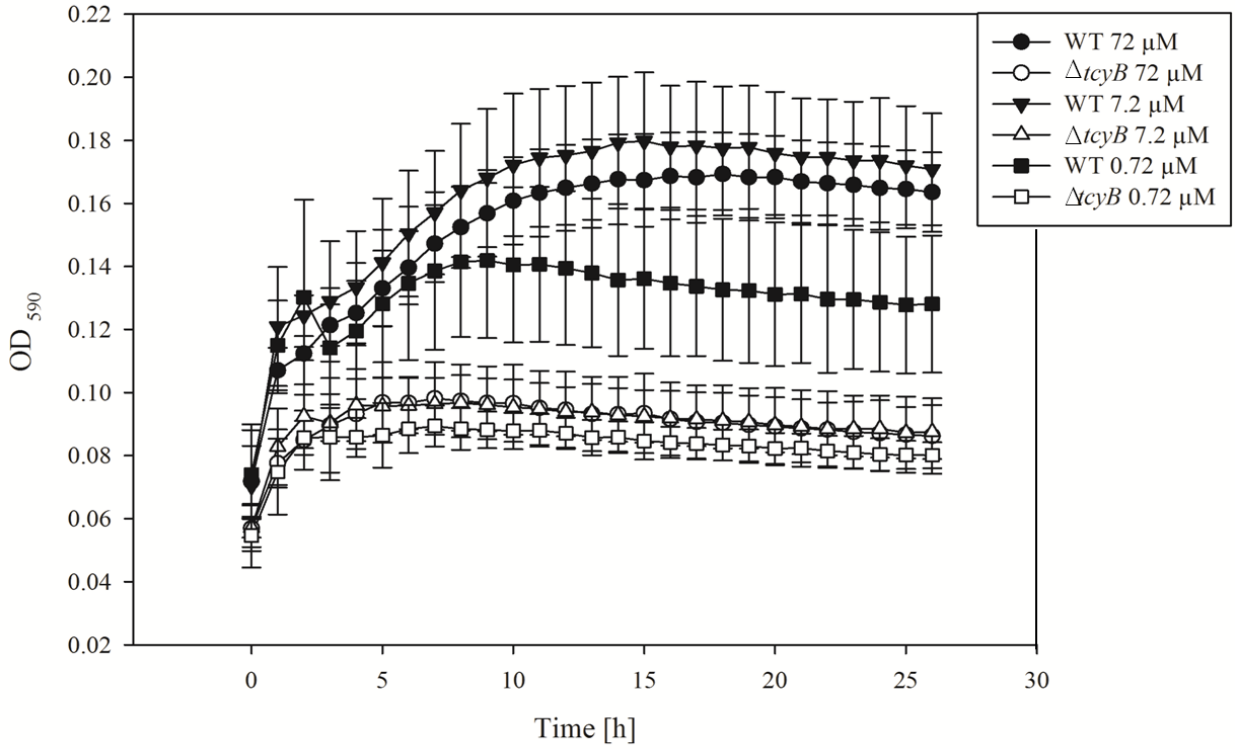


Figure 12: Growth curves of WT and Δ*tcyB* in CDM without cysteine with added cystine (72 µM, 0.72 µM and 0.72 µM). Visible are the mean OD 590 values with corresponding standard deviations of triplicate measurements. Closed symbols label the WT; open symbols flag the Δ*tcyB* mutant.

The growth of the WT was comparable with 7.2 µM and 72 µM of added cystine (Figure 12). The concentration of 0.72 µM cystine was probably to low as OD values decrease. Overall, the WT grew better than Δ*tcyB*. The growth of the mutant was hardly detectable and independent of the concentrations of added cystine.

Looking at the growth curves in CDM with added cysteine (Figure 13) showed a similar picture as above. The WT grew best with 28.5 µM and 0.285 mM added cysteine whereas a decrease of OD values occurred with 2.85 µM cysteine. The Δ*tcyB* mutant grew better with 28.5 µM cysteine which was comparable with the growth of the WT with 2.85 µM of cysteine. The OD values of Δ*tcyB* were even a little higher than for the WT with 0.285 mM of cysteine.

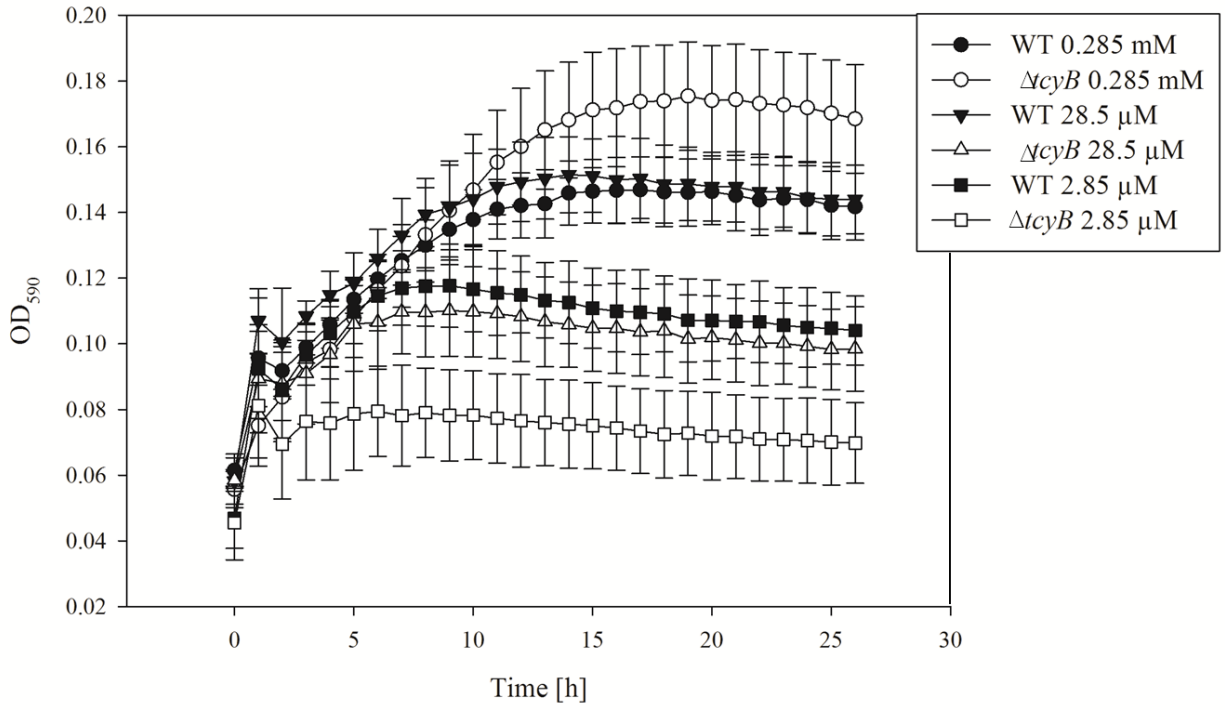
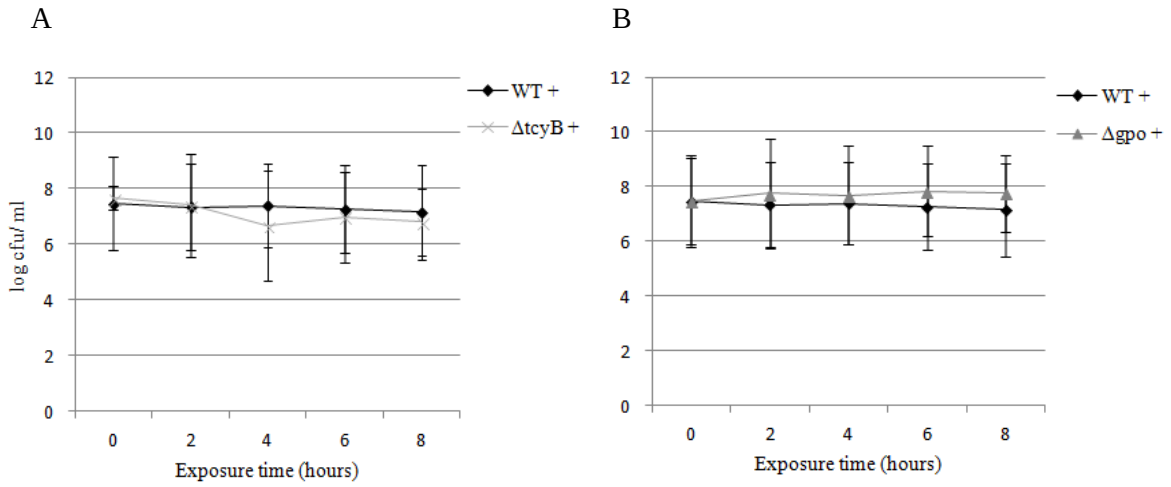


Figure 13: Growth curves of WT and Δ*tcyB* in CDM with cysteine in three different concentrations (0.285 mM, 28.5 µM and 2.85 µM). Visible are the mean OD 590 values with corresponding standard deviations of triplicate measurements. Closed symbols label the WT; open symbols flag the Δ*tcyB* mutant.

4.8 Survivability after H_2O_2 and diamide shock

The sensitivity against peroxide and thiol stress after application of H_2O_2 and diamide to stationary phase grown cells was tested to determine the number of viable cells in mMRS media.

No significant differences between WT, Δ*gpo* and Δ*tcyB* mutants could be observed (Figure 14 A- D). Neither H_2O_2 nor diamide treatment changed the number of viable bacteria. The values ranged from 6.8 log cfu/ mL to 7.6 log cfu/ mL of treated samples and were equal to the values obtained for the untreated samples (data not shown).



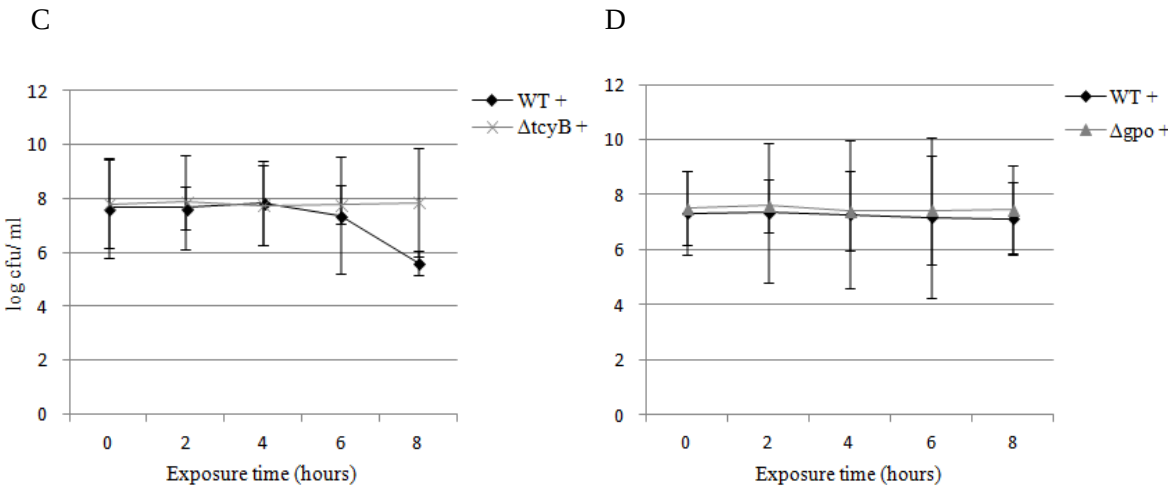


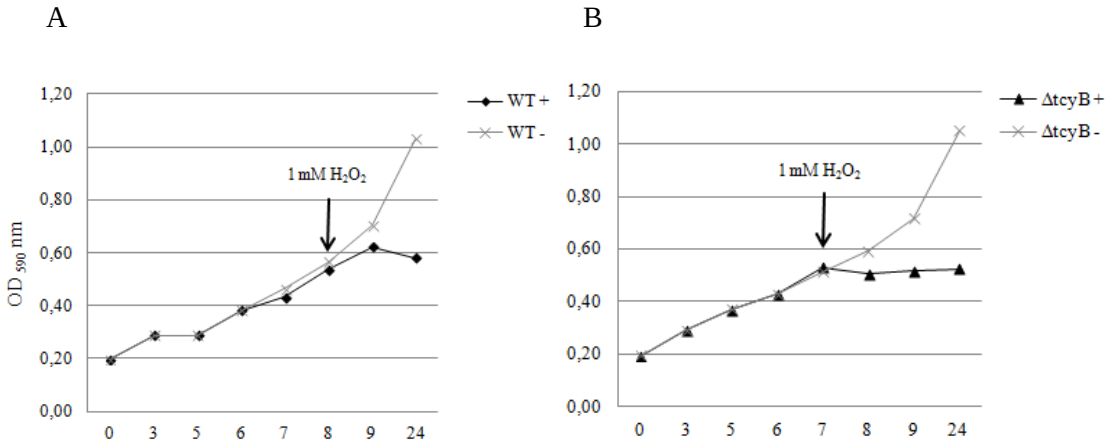
Figure 14: Effect of H_2O_2 (A, B) and diamide (C, D) addition of the survival of viable cells of stationary cultures of WT, Δ*tcyB* and Δ*gpo*. Plotted are the log cfu per mL of treated (+) WT and treated (+) Δ*tcyB* or Δ*gpo* mutant against the exposure time in hours. Shown are the mean values including standard deviations of the cfu determined from triplicate measurements. The survival curves for the untreated samples are not shown as the values are not different to the ones represented here.

4.9 Shock experiments with diamide and H_2O_2

Exponential phase cells (OD 0.5) were shocked with H_2O_2 and diamide for 1 hour to collect samples for thiol group determination and OD measurements. The results of the OD measurements are displayed in Figure 15 (H_2O_2) and Figure 16 (diamide).

As visible in Figure 15 A, the OD of the WT showed an increase after application of H_2O_2 between the eighth and ninth hour and reached a plateau after 9 and 24 hours. The final OD after 24 hours remained at 0.6 for the WT.

The OD curves after H_2O_2 treatment of Δ*tcyB* (15 B) and Δ*gpo* (15 C) were similar. No increase in cell density for Δ*tcyB* and Δ*gpo* could be measured. The OD values remained at ~ 0.5.



C

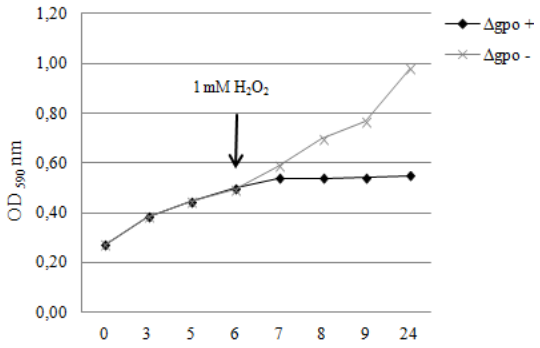
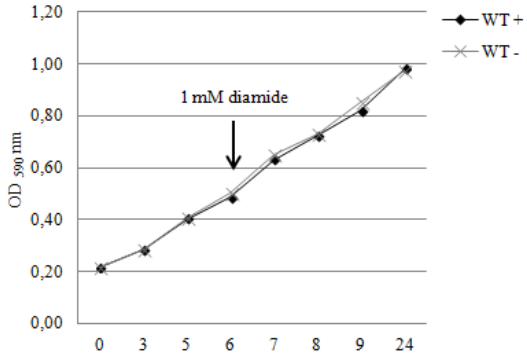


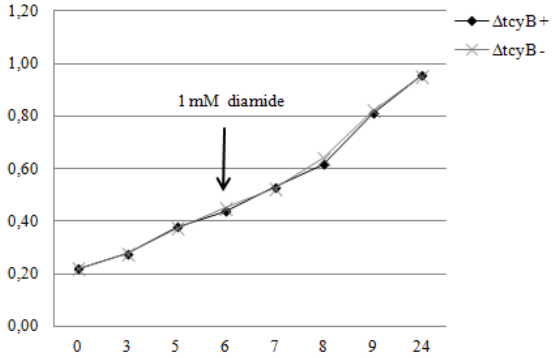
Figure 15: Growth curves of WT (A), Δ*tcyB* (B) and Δ*gpo* (C) after application of 1 mM H_2O_2 determined at different times (T_0, T_3, T_5, T_6, T_7, T_8, T_9 and T24). Shown are mean OD_{590} nm values of the treated (+) and untreated (-) samples against the incubation time in hours. Measurements were conducted in triplicate; standard deviations were omitted due to clear arrangement of the curves.

The effects after application of diamide are visible in Figure 16. Neither the WT (16 D) nor the mutants (16 E, 16 F) showed any differences in OD values of the treated (+) vs. untreated (-) samples. The curves were similar and showed no changes after 1 hour of diamide treatment.

D



E



F

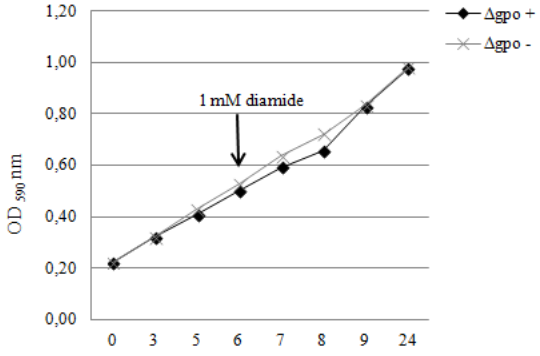


Figure 16: Growth curves of WT (D), Δ*tcyB* (E) and Δ*gpo* (F) after application of 1 mM diamide determined at different times (T_0, T_3, T_5, T_6, T_7, T_8, T_9 and T_{24}). Shown are mean OD_{590} nm values of treated (+) and untreated (-) samples against the incubation time in hours. Measurements were conducted in triplicate; standard deviations were omitted due to clear arrangement of the curves.

4.10 Quantification of extracellular and intracellular thiol groups after H_2O_2 and diamide treatment

Intracellular and extracellular thiol groups were determined to obtain information if the application of H_2O_2 and diamide disturbs the extracellular and/ or intracellular thiol balance in the Δ*gpo* and Δ*tcyB* mutants of *L. sanfranciscensis.*

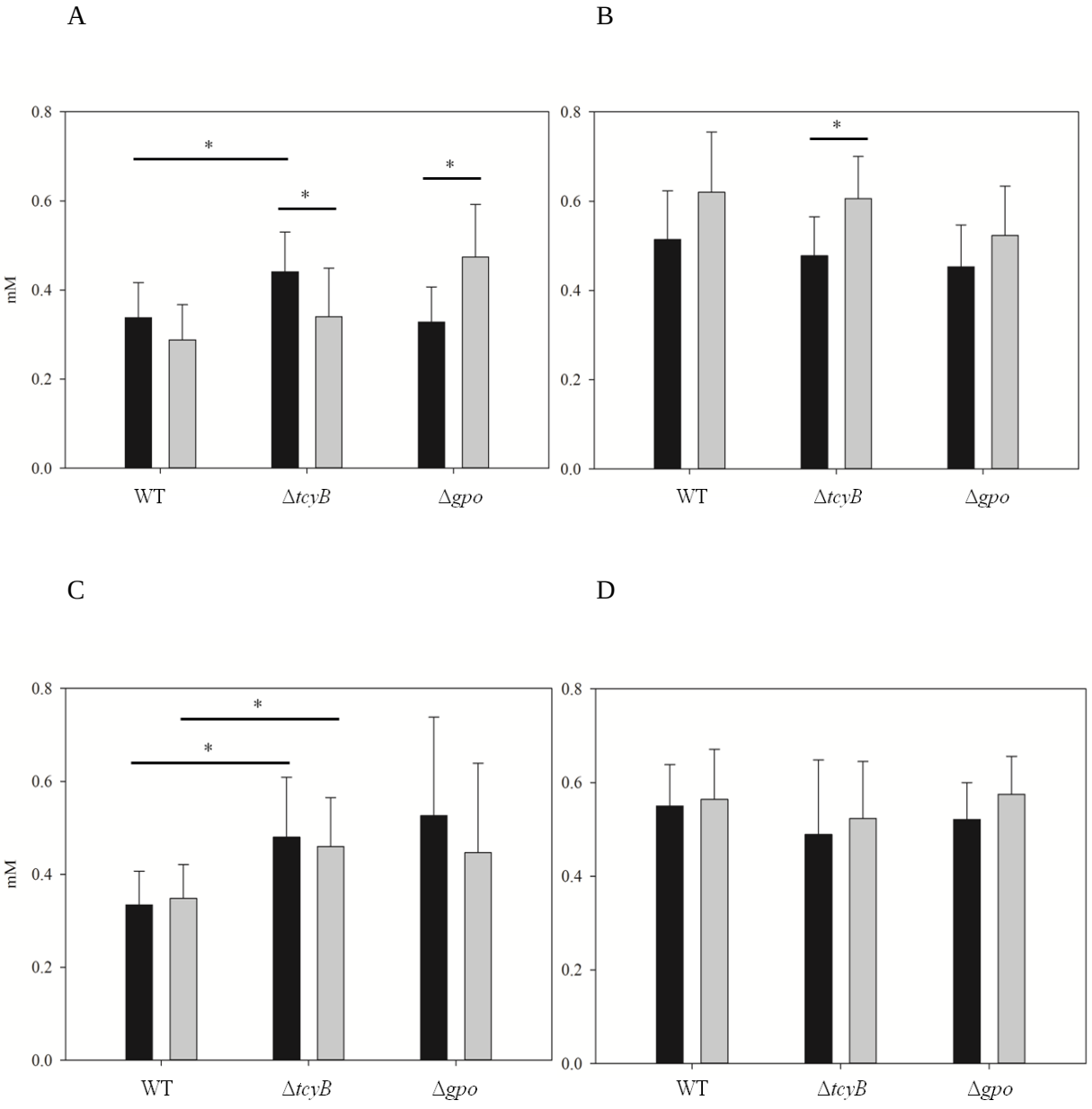


Figure 17: Extracellular and intracellular thiol group quantification of WT, Δ*tcyB* and Δ*gpo*. Plotted are the mean values of triplicate measurements with corresponding standard deviations. Visible are the concentrations of thiols in mM at OD 0.5 after H_2O_2 (A, B) and diamide (C, D) treatment. On the left of each line, the extracellular thiol groups, on the right the quantified intracellular thiol groups in mM are shown. The dark grey bars indicate the treated samples whereas the light grey bars are the control samples. *$p \leq 0.05$

The results of determined extracellular and intracellular thiol groups after H_2O_2 and diamide treatment are depicted in Figure 17.

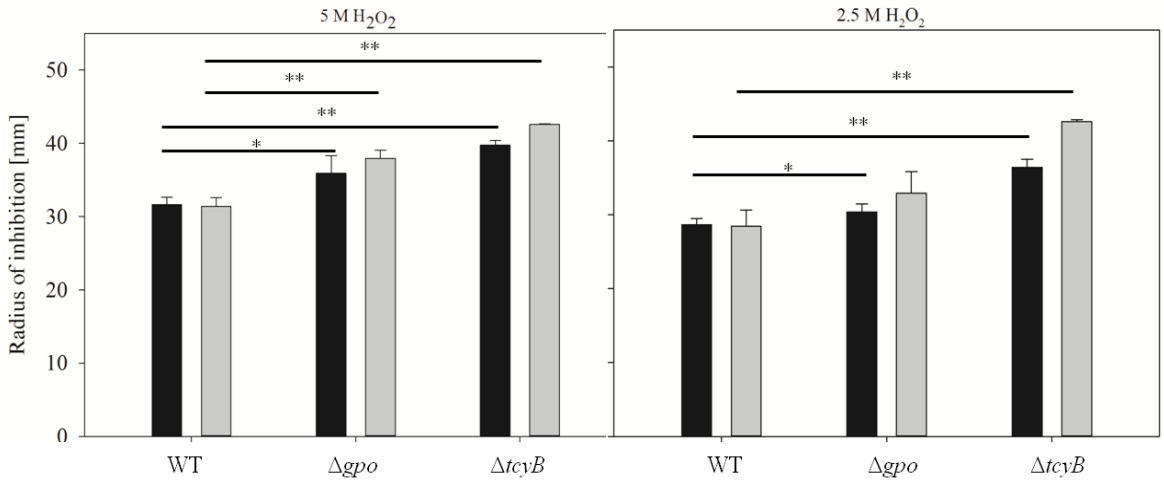
The application of H_2O_2 increased the extracellular thiol groups in the Δ*tcyB* mutant compared with the WT (17 A). The concentration of thiols in the Δ*tcyB* mutant was significantly higher compared to the control. In the Δ*gpo* mutant the extracellular thiol groups were significantly higher compared to the WT (17 A). The intracellular thiol groups after H_2O_2 treatment (17 B) were significantly higher in the control of the Δ*tcyB* mutant compared to the treated samples.

A higher concentration of extracellular thiol groups in the Δ*tcyB* mutant compared with the WT could also be observed in 17 C, although the effect was independent of the diamide treatment. The Δ*gpo* mutant showed no significant differences in extracellular thiol levels compared to the WT (17 C). No significant differences could be observed for the intracellular thiol groups; only the Δ*tcyB* mutant showed a little trend towards lower thiol levels compared to the WT (17 D).

4.11 Sensitivity tests of WT, Δ*gpo* and Δ*tcyB* against oxidants

For testing the sensitivity of WT and mutants, strains were plated onto different media (mMRS, mMRS without cysteine and fructose) with applied H_2O_2, paraquat and diamide each in two different concentrations. The results are displayed in Figure 18.

The effect of applied H_2O_2 showed the most pronounced effects in mMRS and mMRS5 media. The radius of inhibition for Δ*gpo* and Δ*tcyB* was significantly higher compared to the WT in both media types when 5 M H_2O_2 was used (18 A). The effects for Δ*tcyB* were highly significant at both H_2O_2 concentrations (A, B). The sensitivity against H_2O_2 depended also on the used media.



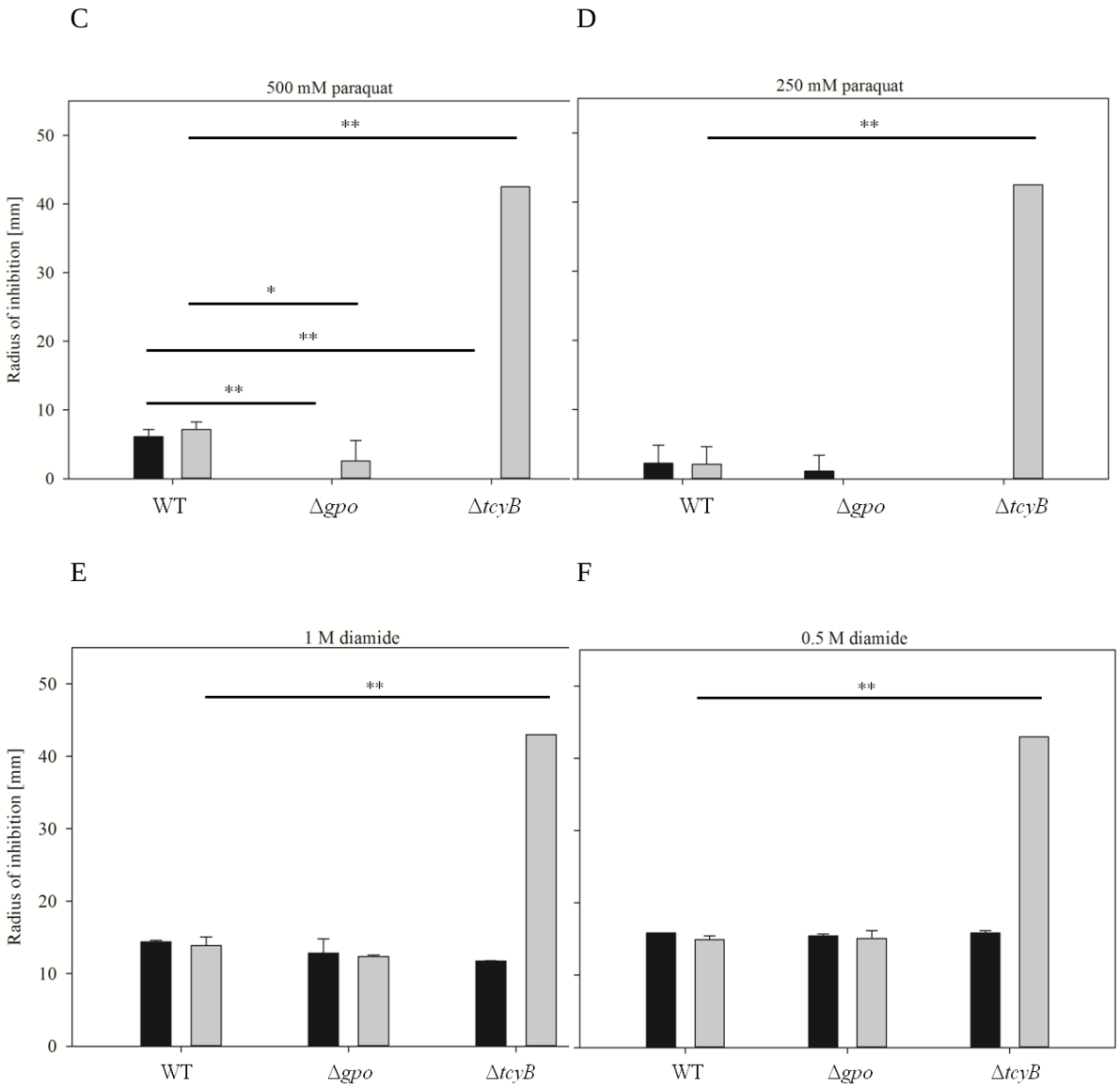


Figure 18: Growth- zone inhibition assay of WT, Δ*gpo* and Δ*tcyB*. The zones of growth inhibition after H_2O_2 (A, B), paraquat (C, D) and diamide (E, F) challenge shown in millimeter (mm) on the y- axis. The black bars indicate the incubation onto mMRS plates, the grey bars onto mMRS5 plates. Corresponding concentrations of the used reagents are labeled on top of the diagrams. The experiment was conducted four times; mean values are displayed with corresponding standard deviations. *$p \leq 0.05$, **$p \leq 0.01$.

The effects of superoxide generating paraquat are visible in Figure 18 C and D. The mutants Δ*gpo* and Δ*tcyB* showed a higher resistance against paraquat as no inhibition zones could be measured in mMRS with 500 mM paraquat (C). The Δ*tcyB* mutant showed a high sensitivity in mMRS5 compared to the WT at both paraquat concentrations (C, D).

The application of diamide showed little effects on the sensitivities of the mutants as depicted in diagrams E and F. The thiol- oxidizing agent had no effect on the Δ*gpo* mutant at both concentrations. In contrast, the Δ*tcyB* mutant showed higher sensitivity in mMRS5 media at both concentrations compared to the WT (E, F).

4.12 Growth response in the presence of different reducing and oxidizing agents

Corrected OD values of WT, Δ*gpo* and Δ*tcyB* after 16 hours of the six different concentrations (100 mM, 40 mM, 20 mM, 10 mM, 5 mM and 1 mM) were taken for analysis. Interaction plots for all concentrations including standard deviations are displayed in Figure 19 (A - F). The corresponding significant p-values between the growths of WT and mutants in the presence of oxidizing and reducing agents are summarized in Table 20.

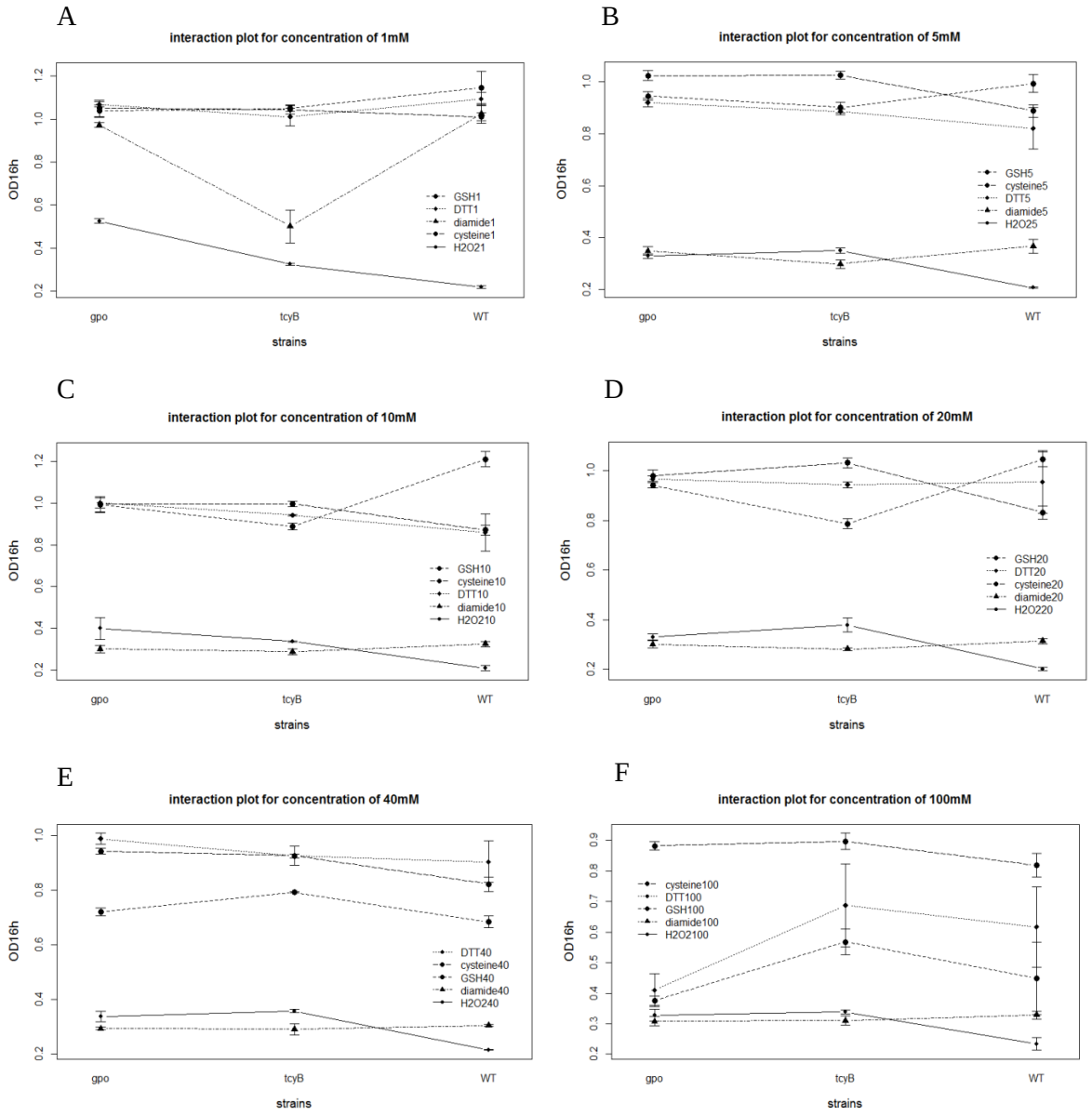


Figure 19: Interaction plots of WT, Δ*gpo* and Δ*tcyB* grown in mMRS media with cysteine, DTT, glutathione, diamide and H_2O_2 at concentrations of 1 mM (A), 5 mM (B), 10 mM (C), 20 mM (D), 40 mM (E) and 100 mM (F). Plotted are the OD_{590} values of triplicate measurements after 16 hours of growth of the mentioned strains on the x- axis with corresponding standard deviations. The five used chemicals are marked in each interaction plot with suitable symbols.

Most pronounced effects could be observed when 1 mM of oxidants were used. The Δ*tcyB* mutant showed significantly lower OD values at 1 mM diamide compared to the OD values of the WT and the Δ*gpo* mutant (A). Further, the Δ*gpo* mutant showed a significantly higher resistance against 1 mM

applied H_2O_2 compared to the WT. It reached OD values of ~ 0.5 whereas only OD values of 0.2 – 0.3 could be seen for the WT and Δ*tcyB* mutant. The same observation could be retrieved from the interaction plot with other concentrations of H_2O_2 (B, C, D, E and F). The OD values of Δ*gpo* and Δ*tcyB* mutant were higher compared to the WT.

Significant higher OD values of Δ*gpo* and Δ*tcyB* mutant were reached after application of 20 mM cysteine compared to the WT (D). The incubation with 20 mM GSH led to significant lower OD values of the Δ*tcyB* mutant compared to the OD values of the WT. The Δ*gpo* mutant showed lower OD values (OD 0.3 – 0.4) in the presence of 100 mM GSH and 100 mM DTT compared to the WT (OD 0.5 – 0.7) (F).

Table 20: Significance values of WT, Δ*gpo* and Δ*tcyB* after 16 hours of growth with different oxidizing and reducing agents. Two- way ANOVA was used to indicate significant effects between the concentrations, used chemicals and strains, Tukey`s HSD test was used to calculate corresponding p-values.

strains	Used chemical and corresponding concentration in mM	p-values
WT: Δ*tcyB*	diamide- 1 mM	0.0000000
Δ*tcyB*: Δ*gpo*	diamide- 1 mM	0.0000000
WT: Δ*gpo*	H_2O_2- 1 mM	0.0001137
Δ*tcyB*: Δ*gpo*	H_2O_2- 1 mM	0.0276948
WT: Δ*tcyB*	H_2O_2- 5 mM	0.0461015
WT: Δ*gpo*	H_2O_2- 10 mM	0.0220590
WT: Δ*tcyB*	GSH- 20 mM	0.0019355
WT: Δ*tcyB*	Cysteine- 20 mM	0.0363688
WT: Δ*tcyB*	H_2O_2- 40 mM	0.0294389

4.13 Fermentations and metabolite analysis of WT, Δ*gpo*, Δ*tcyB* and Δ*nox*

Fermentations with analysis of ORP, pO_2 and pH were conducted to detect differences in ORP development and oxygen reducing abilities of WT and mutants.

The E_h values with corresponding pO_2 values of WT, Δ*gpo*, Δ*tcyB* and Δ*nox* over the fermentation time of 24 hours are displayed in Figure 20.

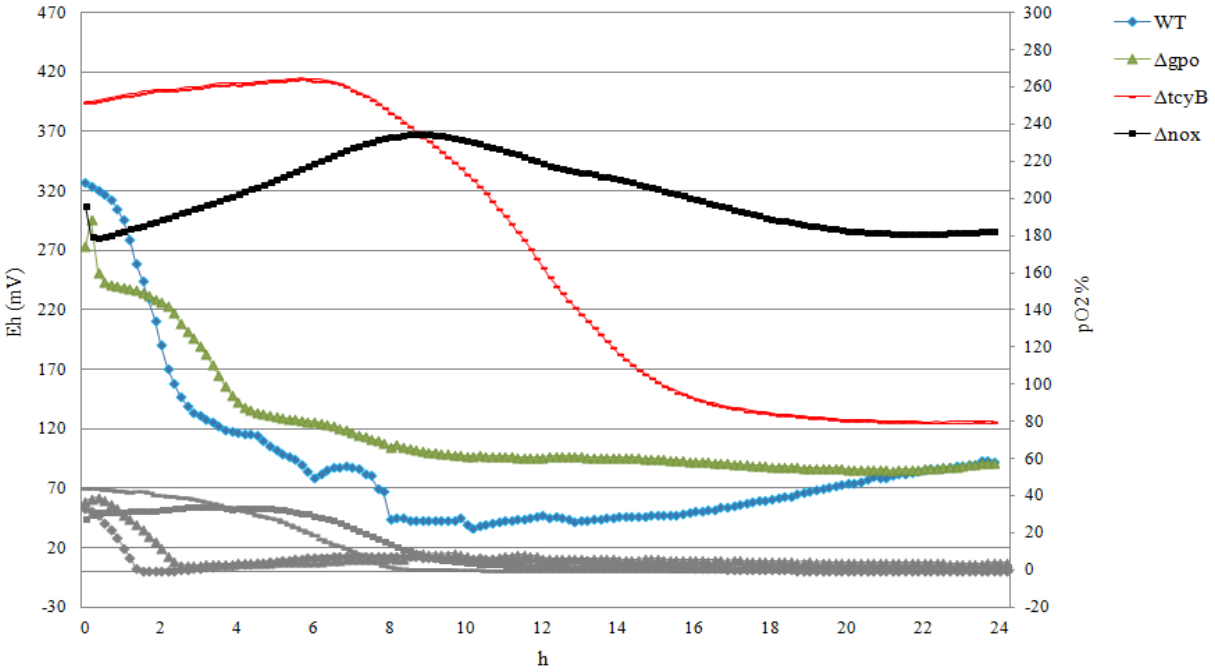


Figure 20: Development of E_h and pO_2 % of WT, Δ*gpo*, Δ*tcyB* and Δ*nox* in mMRS over 24 hours. Visible are the mean values of E_h of triplicate measurements of WT (blue diamond), Δ*gpo* (green triangle), Δ*tcyB* (red bar) and Δ*nox* (black square). The SD values for E_h were omitted due to clear arrangement of the curves. The means of SD values of E_h are 40.4 (WT), 25.9 (Δ*gpo*), 32.2 (Δ*tcyB*) and 29.4 (Δ*nox*). Corresponding mean pO_2 values in % are depicted in grey, the same symbols were used.

Overall, positive E_h values were reached for the four examined strains. The E_h for WT, Δ*gpo* and Δ*nox* started between 270 and 320 mV, for Δ*tcyB* above 370 mV. The curves for WT and Δ*gpo* look similar, however the WT reached lower E_h values especially between hours 4 and 18. In contrast to the WT, the E_h curve for Δ*tcyB* remained stable for the first six hours, before it started to drop between 7 and 14 hours. In the remaining 10 hours, E_h values approached the values for WT and Δ*gpo*. The E_h curve for Δ*nox* was also different to the others. In the first 8 hours, an increase in E_h values (from 280 mV to 370 mV) could be observed before the values dropped to the initial point of approx. 280 mV. The final E_h values were much higher compared to the values for the WT (280 mV vs. 80 mV).

Looking at the pO_2 values, WT and Δ*gpo* showed a similar oxygen consumption rate (Figure 20, Table 21). The mutants Δ*tcyB* and Δ*nox* needed longer to eliminate the oxygen, whereas Δ*nox* had the lowest oxygen reducing capacity.

Retrieving the data concerning the fermentation kinetics (Table 21) revealed no significant differences in V_{mr} and V_{ma} as well as O_{mr} between WT and tested mutants ($p > 0.05$). In all fermentations, V_{mr} occurred after O_{mr} except for Δ*nox*.

Minor differences between the strains can be observed. The highest V_{mr} achieved the WT, followed by Δ*nox*. The time reaching the maximal reduction of Δ*nox* occured at the same time as the maximum acidification after 3.3 h.

The highest oxygen consumption had the WT (- 34 pO_2 %/ h), followed by Δ*gpo* and Δ*tcyB*. The lowest oxygen consumption had Δ*nox* (- 9.8 pO_2 %/ h). All mutants needed more time to reach the maximum oxygen consumption compared to the WT. Especially the mutants Δ*tcyB* and Δ*nox* had lower oxygen consumption values and needed longer to achieve similar values (6.2 h and 7.7 h) as already described above.

The maximum acidification rate had Δ*nox* followed by WT and Δ*gpo*, the lowest value had Δ*tcyB*. Simultaneously, the Δ*nox* mutant reached the maximum acidification before the WT at 3.3 h. The Δ*tcyB* mutant achieved the maximum acidification later than the other strains.

Table 21: Mean values of fermentation kinetics calculated from the reduction V_{mr}, acidification V_{ma} and oxygen consumption O_{mr} rates of WT (n = 3), Δ*gpo* (n = 3), Δ*tcyB* (n = 3) and Δ*nox* (n = 3).

strain	V_{mr} (mV/ h)	T_{mr} (h)	O_{mr} (pO_2%/ h)	T_{or} (h)	V_{ma} (pH unit/ h)	T_{ma} (h)
WT	- 131.8 ± 4.73	2.0	- 34.0 ± 20.19	1.3	- 0.5 ± 0.193	4.0
Δ*gpo*	- 69.14 ± 49.13	3.5	- 25.2 ± 20.27	2.2	- 0.36 ± 0.06	5.7
Δ*tcyB*	- 55.80 ± 12.15	11.7	- 13.6 ± 9.69	6.2	- 0.24 ± 0	8.8
Δ*nox*	- 80.23 ± 145.29	3.3	- 9.8 ± 3.08	7.7	- 1.62 ± 2.55	3.3

The production of organic acids and ethanol of WT and mutants can be taken from Table 22. After 8 hours the Δ*gpo*, Δ*tcyB* and Δ*nox* mutants produced less lactate compared to the WT. After 24 hours Δ*gpo* and Δ*tcyB* reached the values of the WT whereas lactate production for Δ*nox* mutant was still lower. No significant differences between WT and mutants could be seen for acetate production neither after 8 hours nor after 24 hours of growth. The production of ethanol was significantly increased for the Δ*nox* mutant after 8 and 24 hours. The Δ*gpo* mutant showed a slightly increased ethanol production after 24 hours compared to the WT however not as high as Δ*nox*.

Table 22: Metabolite production of WT, Δ*gpo*, Δ*tcyB* and Δ*nox* fermented in mMRS expressed in mM with corresponding standard deviations. *p ≤ 0.05, **p ≤ 0.01.

Strains	Lactate (mM)		Acetate (mM)		Ethanol (mM)	
	8 h	24 h	8 h	24 h	8 h	24 h
WT	73.88 ± 6.37	148.76 ± 5.31	17.65 ± 1.99	19.45 ± 0.53	10.73 ± 2.57	51.40 ± 1.20
Δ*gpo*	42.72* ± 0.09	146.71 ± 2.71	17.32 ± 0.28	19.14 ± 0.34	10.36 ± 1.75	63.11* ± 3.52
Δ*tcyB*	28.36** ± 0.15	148.53 ± 2.28	13.73 ± 0.10	19.22 ± 0.18	9.08 ± 0.85	49.09 ± 11.47
Δ*nox*	19.15** ± 3.27	66.31** ± 3.59	20.56 ± 3.17	21.81 ± 1.55	29.56** ± 1.51	147.10** ± 9.32

The consumption and production of sugars/ sugar alcohols of WT and mutants can be retrieved from Figure 21 and Figure 22. The decrease in fructose of WT, Δ*gpo* and Δ*nox* to very low values after 8 hours was comparable whereas Δ*tcyB* reached the same level not until 24 hours (Figure 21 A). As the production of mannitol depends on fructose consumption, the beginning of the curves in Figure 21 B resembled the values observed in Figure 21 A. The first increase in mannitol production of WT, Δ*gpo*

and Δ*nox* was comparable whereas significant higher mannitol production occurred for Δ*gpo* and Δ*nox* already after 6 hours with maximum values reached in the twelfth hour. The WT and Δ*tcyB* reached final concentrations of 4000 – 5000 µM whereas Δ*gpo* and Δ*nox* ended up with 6500 – 8000 µM of mannitol. The increase in mannitol production over the time for Δ*tcyB* was slower compared to the WT.

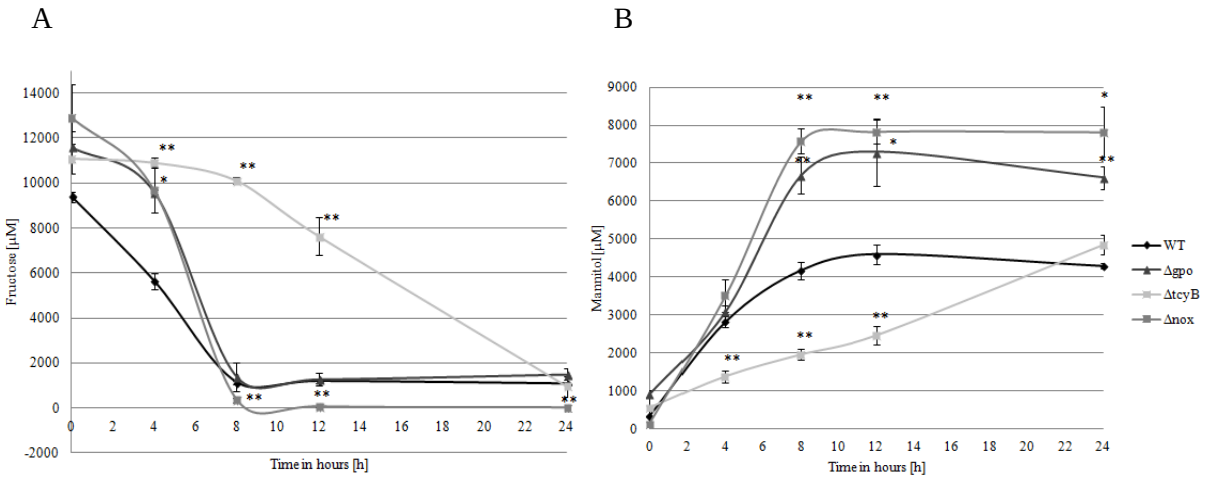


Figure 21: Fructose consumption (A) and mannitol production (B) in µM of WT Δ*gpo*, Δ*tcyB* and Δ*nox*. Visible are the mean values in µM of triplicate measurements with corresponding standard deviations during 24 hrs of fermentation. Samples were taken and measured at time points 0, 4, 8, 12 and 24 hours. *p ≤ 0.05, **p ≤ 0.01.

No significant differences in maltose consumption could be seen when comparing all strains (Figure 22 A). The decline of the curves for Δ*gpo* and Δ*tcyB* was lower compared to the WT and Δ*nox*, however after 24 hours the end concentrations were similar.

As the initial glucose concentrations of the used mMRS differed strongly between the strains (Figure 22 B), conclusions have to be drawn carefully. For the Δ*tcyB* a short increase in glucose concentration could be observed until 8 hours before the concentration dropped. The final concentration of glucose was comparable between the strains.

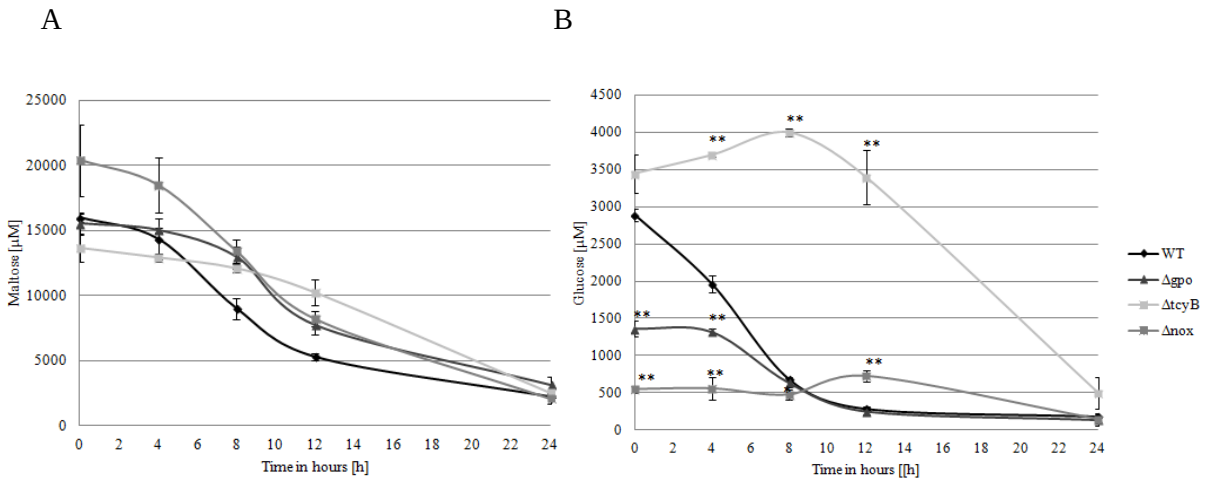


Figure 22: Maltose (A) and glucose (B) consumption in µM of WT Δ*gpo*, Δ*tcyB* and Δ*nox*. Visible are the mean values in µM of triplicate measurements with corresponding standard deviations during 24 hrs of fermentation. Samples were taken and measured at time points 0, 4, 8, 12 and 24 hours. *$p \leq 0.05$, **$p \leq 0.01$.

No significant differences could be obtained from HPLC measurements quantifying amino acids between WT and mutants (results not shown).

4.14 Results of PCR Screening

Besides the characterization of deleted "redox" genes in *L. sanfranciscensis*, another important aim of the present work included the PCR- screening of known "redox" genes, peptidases, dehydrogenases and genes, which are known to participate in reactions of the ETC in LAB. The screening for aroma genes shall give information if some of the used strains also take part in aroma formation.

The results of the PCR- screening are summarized in Table 23. *Lactobacillus* strains with the most positive results included strains of *L. plantarum*, *L. brevis*, *L. pontis* and *L. paracasei*. Strains with less positive results were *L. sanfranciscensis*, *L. spicheri*, *L. paralimentarius*, *Pediococcus pentosaceus*, *L. acidophilus* and *Weissella* (*cibaria*, *confusa*) strains.

Table 23: Results of screening- PCRs for genes coding for thioredoxin reductase (*TrxR*), glutathione peroxidase *(gpo*), thioredoxin-like protein (*ytp*), peptide methionine sulfoxide reductase (*msrA*), spx protein (*spx*), NADH peroxidase (*Npox*), ferredoxin- NADP reductase (*Red*), aminopeptidase C (*pepC*), aminopeptidase E (*pepE*), opep (oligoendopeptidase F), 6- phosphogluconate dehydrogenase (*phgluc*), glyceraldehyde- 3- phosphate dehydrogenase (*gladdDH*), cytochrom B (*cytB*), nitratreduktase (*narH*), NAD(P)H dehydrogenase (*Ndh2*), phenolic acid decarboxylase (*Pdc*), alpha-L- Arabinfuranosidase (*AFN*) and ferulic acid esterase (*Fae*). Grey colored boxes mark "redox genes", green boxes label peptidase genes, blue boxes flag dehydrogenase genes, orange boxes are genes involved in ETC and purple boxes mark aroma genes. Filled boxes indicate negative results, empty boxes positive results.

	Tested strains of Technische Mikrobiologie Weihenstephan (TMW)	**TrxR**	**gpo, gpx**	**ytpP**	**msrA**	**spx**	**Npox**	**Red**	**pepC**	**pepE**	**opep**	**phgluc**	**gladdDH**	**cytB**	**narH**	**Ndh2**	**Pdc**	**AFN**	**Fae**
1	*L. plantarum* TMW 1.1723					■		■					■		■			■	
2	TMW 1.1478					■		■					■					■	
3	TMW 1.1372					■		■					■		■			■	
4	TMW 1.701					■		■					■					■	
5	TMW 1.460					■		■					■					■	
6	TMW 1.1732					■		■					■					■	
7	TMW 1.1							■		■								■	■
8	TMW 1.702					■		■					■					■	
9	TMW 1.1809					■		■										■	■
10	TMW 1.1237														■			■	
11	TMW 1.124												■					■	
12	TMW 1.1204					■							■					■	■
13	*L. sanfran.* TMW 1.1304		■		■	■	■				■	■	■	■	■		■		■
14	TMW 1.1461		■		■	■	■				■	■	■	■	■	■	■		■
15	TMW 1.398		■			■		■			■	■	■			■		■	
16	TMW 1.377		■		■	■	■				■	■	■	■	■	■	■		
17	TMW 1.392		■		■	■	■				■	■	■	■	■	■	■		■
18	TMW 1.728					■		■				■	■		■			■	
19	TMW 1.53		■		■		■				■	■	■	■	■		■		
20	*L. brevis* TMW 1.384		■		■	■	■				■	■		■	■		■		■
21	TMW 1.791		■			■	■	■		■	■	■		■	■		■	■	■
22	TMW 1.57				■		■	■								■			■
23	TMW 1.1785				■			■								■			■
24	TMW 1.1369				■			■								■			■

	Tested strains of Technische Mikrobiologie Weihenstephan (TMW)	TrxR	gpo, gpx	ytpP	msrA	spx	Npox	Red	pepC	pepE	opep	phgluc	gladDH	cytB	narH	Ndh2	Pdc	AFN	Fae
25	TMW 1.313				■		■	■				■				■			■
26	TMW 1.1326				■			■								■			■
27	TMW 1.6		■		■		■	■								■			■
28	TMW 1.1807				■		■	■								■			■
29	TMW 1.100				■	■		■								■			■
30	TMW 1.1786				■	■		■								■			■
31	TMW 1.1787				■	■		■								■			■
32	*L. panis* TMW 1.648					■		■					■					■	
33	*L. pontis* TMW 1.1086		■		■	■	■	■			■	■	■	■	■	■		■	
34	TMW 1.1300		■		■	■	■	■			■	■	■	■	■	■		■	■
35	TMW 1.1301				■	■		■			■	■		■		■		■	■
36	TMW 1.56				■			■								■			■
37	*L. spicheri* TMW 1.1225			■	■	■	■	■		■	■	■			■		■		
38	TMW 1.1233			■	■	■	■	■		■	■	■			■		■		
39	TMW 1.1226			■	■	■	■	■		■	■	■	■	■	■	■	■		
40	*L. paralim.* TMW 1.1725				■	■		■			■	■		■	■	■	■	■	
41	TMW 1.1234				■	■		■		■	■	■		■	■		■	■	
42	TMW 1.1726	■			■			■			■	■			■	■	■	■	■
43	TMW 1.256	■	■		■			■			■	■		■	■		■	■	■
44	TMW 1.1235			■	■			■			■	■		■	■	■	■	■	
45	*L. paracasei* TMW 1.1434				■			■		■		■					■	■	
46	TMW 1.1724				■			■		■		■					■	■	
47	*L. casei* TMW 1.1462				■			■		■		■					■	■	■
48	*L. paracasei* TMW 1.1305				■			■		■		■					■	■	■
49	TMW 1.1213				■			■		■		■					■	■	■
50	TMW 1.304				■			■				■					■	■	■
51	*L. ferment.* TMW 1.1727	■	■		■			■		■	■	■			■			■	■
52	TMW 1.1835	■	■		■			■		■	■	■			■	■		■	■
53	TMW 1.1836	■	■		■			■		■	■	■				■		■	■
54	TMW 1.1837	■	■	■	■			■		■	■	■				■		■	■
55	TMW 1.1788				■			■											■
56	*L. sakei* TMW 1.704		■	■	■			■			■			■	■			■	■
57	TMW 1.1239		■	■	■			■			■			■	■		■	■	■
58	TMW 1.705		■		■			■			■			■	■		■	■	■
59	*L. mindensis* TMW 1.1206			■	■	■		■		■	■	■		■	■			■	■
60	*P. pentos.* TMW 2.6		■		■		■	■		■	■	■		■	■	■		■	■
61	TMW 2.74		■		■		■	■		■	■	■		■	■	■		■	■
62	TMW 2.149		■		■	■		■		■	■		■	■	■	■		■	■
63	TMW 2.1036		■		■		■	■		■	■	■			■		■	■	■
64	TMW 2.8		■		■		■	■		■	■	■		■	■			■	■
65	*W. cibaria* TMW 2.1039			■	■	■	■	■		■	■				■		■		■
66	TMW 2.1333			■	■	■	■	■	■	■			■		■		■		■
67	*W. confusa* TMW 2.1034		■	■	■	■		■	■	■	■				■			■	■
68	*L. reuteri* TMW 1.106		■		■	■		■		■	■	■	■					■	

	Tested strains of Technische Mikrobiologie Weihenstephan (TMW)	TrxR	gpo, gpx	ytpP	msrA	spx	Npox	Red	pepC	pepE	opep	phgluc	gladDH	cytB	narH	Ndh2	Pdc	AFN	Fae
69	*L. farciminis* TMW 1.68																		
70	*L. delbrueckii* TMW 1.58																		
71	*L. curvatus* TMW 1.624																		
72	*L. acidophilus* TMW 1.18																		
73	*L. graminis* TMW 1.1174																		
74	*L. perolens* TMW 1.500																		
75	*Leucon. holzapfelii* TMW 2.813																		
76	*E. faecalis* TMW 2.630																		

Based on the number of positive PCR results for "redox" genes, peptidases, dehydrogenases and genes involved in ETC reactions, strains were classified into 5 groups. Each of the groups contained a strain with most positive PCR results; the other with least positive PCR results (grey boxes in Table 23). Different growth responses in the presence of H_2O_2 and diamide were checked to get first information concerning a possible correlation between the presence of tested genes and its tolerance against oxidants.

The results of the growth tests are summarized in Table 24. The two *L. plantarum* (TMW 1.1723, TMW 1.1237) strains showed no different growth behavior, the same could be observed for the two strains of *L. sanfranciscensis* (TMW 1.392; TMW 1.728) and *L. fermentum* (TMW 1.1835; TMW 1.1788). The results for *L. brevis* and *L. pontis* overlapped with the results received from PCR-screening. The strains *L. brevis* TMW 1.791 and *L. pontis* TMW 1.1300 showed a lower tolerance in the presence of H_2O_2 and diamide compared to *L. brevis* TMW 1.1326 and *L. pontis* TMW 1.56.

Table 24: Results of the growth tests of different concentrations of H_2O_2 and diamide of the 5 groups determined before. Each of the groups comprises of a *Lactobacillus* species with the highest number; the other with the lowest number of positive PCR signals as visible in Table 23. Growth was assessed qualitatively in which [1] determines growth and [0] no growth at the corresponding concentrations.

Results of growth tests with oxidizing agents		100 mM H_2O_2	20 mM H_2O_2	10 mM H_2O_2	1 mM H_2O_2	20 mM diamide	10 mM diamide	1 mM diamide	sum	positive PCR signals
1. *L. plantarum*	TMW 1.1723	0	0	0	1	1	1	1	**4**	11
	TMW 1.1237	0	0	0	1	1	1	1	**4**	14
2. *L. sanfranciscensis*	TMW 1.392	0	0	0	1	0	0	0	**1**	5
	TMW 1.728	0	0	0	0	0	0	1	**1**	10
3. *L. brevis*	TMW 1.791	0	0	0	1	0	1	1	**3**	6
	TMW 1.1326	0	0	0	1	1	1	1	**4**	12
4. *L. pontis*	TMW 1.1300	0	0	0	0	0	0	1	**1**	4
	TMW 1.56	0	0	0	1	0	0	1	**2**	12
5. *L. fermentum*	TMW 1.1835	0	0	0	1	1	1	1	**4**	6
	TMW 1.1788	0	0	0	1	1	1	1	**4**	13

4.15 Results of transcriptional analysis using microarray

4.15.1 Aim and experimental setup

Using microarray analysis, the transcriptional response of *L. sanfranciscensis* TMW 1.1304 was studied using conditions, which typically occur in sourdough environments. These included the following: presence of electron acceptors (oxygen, fructose) and yeast (*C. humilis*). Aerobic conditions and the addition of fructose to the media should give information which genes participate in redox reactions if different electron acceptors were present. The incubation with *C. humilis* should answer the question which "redox genes" were differently expressed and if *L. sanfranciscensis* was positively or negatively affected during co- cultivation. Anaerobic incubation resembled the control condition.

4.15.2 Determination of RNA quantity and quality

The quantity and quality of the extracted RNA samples was checked using Nanodrop (Table 25) and Bioanalyzer (electropherograms see Appendix A 16).

Table 25: Quantification of isolated RNA samples (anaerobic, aerobic, Fructose, 1/ 10 and 1/ 100 with *Candida humilis* (C)) from Nanodrop measurement. Samples were diluted 1/ 10 before measurement. The totals RNA in ng/ µL and quality parameters (260/ 280 and 260/ 230) values were determined.

Sample ID	ng/ µL	A 260	A 280	260/ 280	260/ 230
Anaerobic 1/ 10	648.10	16.20	7.33	2.21	2.50
Aerobic 1/ 10	734.56	18.36	8.22	2.23	2.56
Fructose 1/ 10	880.40	22.01	9.91	2.22	2.54
C_1:10 1/ 10	275.98	6.90	3.14	2.20	2.57
C_1:100 1/ 10	581.87	14.54	6.57	2.21	2.56

Nanodrop analysis as well as Bioanalyzer results revealed that RNA quality and quantity was sufficient for further processing.

4.15.3 Expression values after quantification

Significant differentially expressed genes during the different tested conditions are arranged in Table 26 (Aerobic incubation), Table 27 (addition of Fructose) and Table 28 (co- cultivation with *Candida humilis*). Additionally to the data in Table 28, the change in gene expression of *L. sanfranciscensis* TMW 1.1304 incubated with *Candida humilis* TMW 3.191 was visualized in a metabolic pathway in Figure 23.

Table 26: Differentially expressed genes expressed as log2 fold changes during aerobic incubation of *L. sanfranciscensis* TMW 1.1304 ordered alphabetically by COG functional categories. Anaerobic conditions were used as reference condition and set as 1. Up- regulated genes are shaded in grey, downregulated genes are left blank. Genes which were also differentially expressed during incubation with fructose are marked bold. Differentially expressed genes which are denominated as "hypothetical proteins" or "pseudogenes" as retrieved from gene annotation were omitted.

LSA number	COG functional category	Aerobic log2 fold changes	Up/down	gene name
LSA_00330	Amino Acid Metabolism	1.7043	1	aminopeptidase
LSA_00420	Amino Acid Metabolism	0.5993	- 1	Diaminopimelate epimerase
LSA_00430	Amino Acid Metabolism	0.606	- 1	aspartokinase
LSA_00490	Amino Acid Metabolism	0.6239	- 1	aspartate- semialdehyde dehydrogenase
LSA_00870	**Amino Acid Metabolism**	**0.6468**	**- 1**	**aminotransferase A**
LSA_04660	**Amino Acid Metabolism**	**0.4425**	**- 1**	**Homocysteine S- methyltransferase**
LSA_12900	Amino Acid Metabolism	1.6976	1	glycerate kinase 2
LSA_01530	**Carbohydrate Metabolism**	**0.6536**	**- 1**	**beta- phosphoglucomutase**
LSA_01770	Carbohydrate Metabolism	1.7087	1	oligo- 1,6- glucosidase
LSA_02830	**Carbohydrate Metabolism**	**0.6336**	**- 1**	**ribokinase**
LSA_04670	Carbohydrate Metabolism	2.2737	1	L- 2- hydroxyisocaproate dehydrogenase
LSA_05480	**Carbohydrate Metabolism**	**0.6434**	**- 1**	**glucosamine- - fructose- 6- phosphate aminotransferase**
LSA_11920	Carbohydrate Metabolism	0.538	- 1	acetyl- CoA carboxylase carboxyl transferase
LSA_12040	Carbohydrate Metabolism	0.6159	- 1	Fumarate hydratase class II
LSA_12680	Cellular processes	1.6135	1	ATP- dependent Clp protease ATP- binding subunit
LSA_12130	Central intermediary metabolism	2.2485	1	Glyoxal reductase
LSA_09450	Energy Metabolism	1.5196	1	ATP synthase subunit c
LSA_01280	**Genome plasticity**	**0.4475**	**- 1**	**transposase insK for insertion sequence element**
LSA_02280	**Genome plasticity**	**0.6283**	**- 1**	**transposase insK for insertion sequence element**
LSA_12430	Genome plasticity	0.6031	- 1	transposase insK for insertion sequence element
LSA_2p00550	**Genome plasticity**	**1.5206**	**1**	**Transposase for insertion sequence element**
LSA_07400	**Lipid Metabolism**	**0.6134**	**- 1**	**glycerol- 3- phosphate acyltransferase PlsY**
LSA_11910	Lipid Metabolism	0.5416	- 1	acetyl- CoA carboxylase carboxyl transferase
LSA_11940	Lipid Metabolism	0.5357	- 1	(3R)- hydroxymyristoyl- ACP dehydratase
LSA_11960	Lipid Metabolism	0.6313	- 1	3- oxoacyl- ACP synthase
LSA_11970	Lipid Metabolism	0.5916	- 1	3- oxoacyl- ACP reductase
LSA_11980	Lipid Metabolism	0.5723	- 1	[acyl- carrier- protein] S- malonyltransferase
LSA_11990	Lipid Metabolism	0.6078	- 1	acyl carrier protein
LSA_12010	Lipid Metabolism	0.653	- 1	(3R)- hydroxymyristoyl- ACP dehydratase
LSA_00280	Membrane Transport	1.7772	1	oligopeptide transport ATP- binding protein oppD
LSA_00290	Membrane Transport	2.191	1	oligopeptide transport ATP- binding protein oppF
LSA_00300	Membrane Transport	2.6472	1	oligopeptide transport system permease oppB
LSA_00310	Membrane Transport	1.9903	1	oligopeptide transport system permease oppC
LSA_00320	Membrane Transport	2.2859	1	oligopeptide- binding protein oppA
LSA_00940	Membrane Transport	0.5636	- 1	D- methionine transport system substrate- binding protein
LSA_02790	Membrane Transport	0.5762	- 1	amino acid permease

LSA number	COG functional category	Aerobic log2 fold changes	Up/down	gene name
LSA_04650	**Membrane Transport**	**1.7185**	**1**	**amino acid permease**
LSA_05640	**Membrane Transport**	**0.5699**	**- 1**	**cobalt import ATP- binding protein cbiO 2**
LSA_05660	**Membrane Transport**	**0.6123**	**- 1**	**cobalt/nickel transport system permease protein**
LSA_11030	Membrane Transport	0.597	- 1	cobalt/nickel transport system permease protein
LSA_13190	Membrane Transport	1.737	1	phosphonate transport system substrate-binding protein
LSA_01900	Metabolism of Cofactors and Vitamins	0.632	- 1	methylenetetrahydrofolate reductase (NADPH)
LSA_11890	Metabolism of Cofactors and Vitamins	0.3898	- 1	BirA family transcriptional regulator, biotin operon repressor / biotin- [acetyl- CoA-carboxylase] ligase
LSA_12060	**Metabolism of Cofactors and Vitamins**	**0.5161**	**- 1**	**4'- phosphopantetheinyl transferase**
LSA_00350	Nucleotide Metabolism	0.4773	- 1	2',3'- cyclic- nucleotide 2'- phosphodiesterase
LSA_01810	Nucleotide Metabolism	1.6935	1	GMP synthase
LSA_12870	Nucleotide Metabolism	1.968	1	GMP reductase
LSA_1p00080	**Nucleotide Metabolism**	**1.9469**	**1**	**Adenine deaminase 1**
LSA_02010	Other	0.5607	- 1	nrdI-like protein
LSA_13160	**Other**	**0.3265**	**- 1**	**Hemolysin- 3**
LSA_00930	Protein fate	0.5624	- 1	peptidase T
LSA_03230	**Protein fate**	**0.6095**	**- 1**	**serine/threonine exchanger**
LSA_05190	Replication and Repair	1.5031	1	UvrABC system protein A
LSA_07160	Transcription	0.6399	- 1	RNA methyltransferase ypsC
LSA_03720	Translation and protein synthesis	0.5855	- 1	ribosomal RNA small subunit methyltransferase
LSA_04620	**Translation and protein synthesis**	**1.7749**	**1**	**50S ribosomal protein L7/L12**
LSA_06490	Translation and protein synthesis	0.6505	- 1	tRNA Delta(2)- isopentenylpyrophosphate transferase
LSA_07930	**Translation and protein synthesis**	**0.654**	**- 1**	**50S ribosomal protein L11 methyltransferase**
LSA_13510	Translation and protein synthesis	0.5646	- 1	tRNA modification GTPase mnmE

Table 27: Differentially expressed genes expressed as log2 fold changes during incubation with fructose of *L. sanfranciscensis* TMW 1.1304 ordered alphabetically by COG functional categories. Anaerobic conditions were used as reference condition and set as 1. Up- regulated genes are shaded in grey, downregulated genes are left blank. Genes which were also differentially expressed during aerobic incubation are marked bold. Differentially expressed genes which are denominated as "hypothetical proteins" or "pseudogenes" as retrieved from gene annotation were omitted.

LSA number	COG functional category	Fructose log2 fold changes	Up/down	gene name
LSA_00870	**Amino Acid Metabolism**	**1.7333**	**1**	**aminotransferase A**
LSA_04660	**Amino Acid Metabolism**	**1.646**	**1**	**Homocysteine S- methyltransferase**
LSA_05370	Amino Acid Metabolism	0.5581	- 1	phosphate acetyltransferase
LSA_00660	Carbohydrate Metabolism	1.7381	1	6- phosphogluconate dehydrogenase
LSA_01530	**Carbohydrate Metabolism**	**0.5382**	**- 1**	**beta- phosphoglucomutase**
LSA_02830	**Carbohydrate Metabolism**	**0.5221**	**- 1**	**ribokinase**
LSA_05480	**Carbohydrate Metabolism**	**0.5385**	**- 1**	**glucosamine- - fructose- 6- phosphate aminotransferase**
LSA_12190	Carbohydrate Metabolism	0.594	- 1	alcohol dehydrogenase
LSA_12980	Carbohydrate Metabolism	0.6311	- 1	citrate lyase subunit alpha

LSA number	COG functional category	Fructose log2 fold changes	Up/down	gene name
LSA_10170	Cellular processes	0.619	- 1	S- adenosyl- L- methionine- dependent methyltransferase
LSA_05320	Folding, Sorting and Degradation	0.6432	- 1	ribonuclease R 1
LSA_01280	**Genome plasticity**	**0.5267**	**- 1**	**transposase insK for insertion sequence element**
LSA_02280	**Genome plasticity**	**0.6597**	**- 1**	**transposase insK for insertion sequence element**
LSA_2p00110	Genome plasticity	0.543	- 1	transposase
LSA_2p00550	**Genome plasticity**	**1.6931**	**1**	**Transposase for insertion sequence element**
LSA_09330	Glycan Biosynthesis and Metabolism	0.6192	- 1	D- alanine- D- alanine ligase
LSA_07400	**Lipid Metabolism**	**0.5987**	**- 1**	**glycerol- 3- phosphate acyltransferase PlsY**
LSA_12070	Lipid Metabolism	0.5884	- 1	acyl carrier protein
LSA_00850	Membrane Transport	0.3501	- 1	D- methionine transport system substrate-binding protein
LSA_02730	Membrane Transport	0.5242	- 1	ribose uptake protein rbsU
LSA_04650	**Membrane Transport**	**5.606**	**1**	**amino acid permease**
LSA_05640	**Membrane Transport**	**0.6365**	**- 1**	**cobalt import ATP- binding protein cbiO 2**
LSA_05660	**Membrane Transport**	**0.6443**	**- 1**	**cobalt/nickel transport system permease protein**
LSA_13030	Membrane Transport	0.5759	- 1	citrate- sodium symporter
LSA_07110	Metabolism of Cofactors and Vitamins	0.6437	- 1	1,4- dihydroxy- 2- naphthoate octaprenyltransferase
LSA_12060	**Metabolism of Cofactors and Vitamins**	**0.5267**	**- 1**	**4'- phosphopantetheinyl transferase**
LSA_08430	Nucleotide Metabolism	0.6448	- 1	Deoxyguanosine kinase
LSA_1p00080	**Nucleotide Metabolism**	**1.5236**	**1**	**Adenine deaminase 1**
LSA_04140	Other	1.5761	1	multidrug resistance protein mdtG
LSA_13160	**Other**	**0.5192**	**- 1**	**Hemolysin- 3**
LSA_2p00170	Other	1.514	1	Protein crcB-like protein 1
LSA_03230	**Protein fate**	**0.6266**	**- 1**	**serine/threonine exchanger**
LSA_02420	Regulatory functions	0.5932	- 1	regulatory protein spx
LSA_08880	Regulatory functions	0.6424	- 1	catabolite control protein A
LSA_05360	Replication and Repair	0.4925	- 1	uracil- DNA glycosylase
LSA_12960	Signal Transduction	0.5298	- 1	protein citXG
LSA_12990	Signal Transduction	0.6238	- 1	citrate lyase subunit beta
LSA_04620	**Translation and protein synthesis**	**1.7588**	**1**	**50S ribosomal protein L7/L12**
LSA_05840	Translation and protein synthesis	0.5362	- 1	30S ribosomal protein S14
LSA_06310	Translation and protein synthesis	0.6578	- 1	50S ribosomal protein L32
LSA_07930	**Translation and protein synthesis**	**0.6359**	**- 1**	**50S ribosomal protein L11 methyltransferase**
LSA_11170	Translation and protein synthesis	1.6157	1	50S ribosomal protein L18
LSA_11230	Translation and protein synthesis	1.5344	1	50S ribosomal protein L14
LSA_11240	Translation and protein synthesis	1.8691	1	30S ribosomal protein S17
LSA_11270	Translation and protein synthesis	1.5488	1	30S ribosomal protein S3
LSA_11300	Translation and protein synthesis	1.6819	1	50S ribosomal protein L2

LSA number	COG functional category	Fructose log2 fold changes	Up/down	gene name
LSA_11310	Translation and protein synthesis	1.6253	1	50S ribosomal protein L23
LSA_11330	Translation and protein synthesis	1.6477	1	50S ribosomal protein L3
LSA_02080	Xenobiotics Biodegradation and Metabolism	0.533	- 1	aldehyde- alcohol dehydrogenase 2

Table 28: Differentially expressed genes expressed as log2 fold changes during incubation with *Candida humilis* (1/ 10 and 1/ 100) of *L. sanfranciscensis* TMW 1.1304 ordered alphabetically by COG functional categories. Anaerobic conditions were used as reference condition and set as 1. Up- regulated genes are shaded in grey, downregulated or not regulated genes are left blank. Differentially expressed genes which are denominated as "hypothetical proteins" or "pseudogenes" as retrieved from gene annotation were omitted.

LSA number	COG functional category	1/ 100 Candida Log2 fold changes	1/ 10 Candida Log2 fold changes	Up/down	gene name
LSA_00330	Amino Acid Metabolism	1.5575	2.623	1	aminopeptidase
LSA_00870	Amino Acid Metabolism	0.923	0.5813	- 1	aminotransferase A
LSA_04660	Amino Acid Metabolism	0.7435	0.4433	- 1	Homocysteine S- methyltransferase
LSA_07350	Amino Acid Metabolism	1.0277	1.9945	1	peptide methionine sulfoxide reductase
LSA_07360	Amino Acid Metabolism	1.0668	2.0955	1	peptide methionine sulfoxide reductase
LSA_08580	Amino Acid Metabolism	1.0007	1.5493	1	aminotransferase A
LSA_09790	Amino Acid Metabolism	0.7578	2.1507	0	glutathione peroxidase
LSA_10470	Amino Acid Metabolism	0.8549	0.4943	- 1	argininosuccinate synthase
LSA_12900	Amino Acid Metabolism	1.0275	2.2412	1	glycerate kinase 2
LSA_00860	Carbohydrate Metabolism	0.8536	0.5362	- 1	D- lactate dehydrogenase
LSA_01530	Carbohydrate Metabolism	0.7706	0.583	- 1	beta- phosphoglucomutase
LSA_01770	Carbohydrate Metabolism	1.175	2.6919	1	oligo- 1,6- glucosidase
LSA_02830	Carbohydrate Metabolism	0.7096	0.6156	- 1	ribokinase
LSA_04670	Carbohydrate Metabolism	1.1575	3.3204	1	L- 2- hydroxyisocaproate dehydrogenase (malate DH)
LSA_10640	Carbohydrate Metabolism	0.658	0.5927	- 1	gluconokinase
LSA_10990	Carbohydrate Metabolism	1.0426	1.9668	1	D- lactate dehydrogenase
LSA_11920	Carbohydrate Metabolism	0.623	0.4125	- 1	acetyl- CoA carboxylase carboxyl transferase
LSA_11950	Carbohydrate Metabolism	0.6581	0.3995	- 1	acetyl- CoA carboxylase biotin carboxyl carrier protein
LSA_09310	Cellular processes	1.2809	1.915	1	universal stress protein
LSA_10410	Cellular processes	0.9966	1.5128	0	ATP- dependent Clp protease ATP- binding subunit
LSA_12680	Cellular processes	1.1821	2.5252	1	ATP- dependent Clp protease ATP- binding subunit
LSA_12130	Central intermediary metabolism	1.3178	3.5219	1	Glyoxal reductase
LSA_04980	Folding, Sorting and Degradation	1.1059	1.9091	1	molecular chaperone GroES
LSA_04990	Folding, Sorting and Degradation	0.9989	1.9172	0	molecular chaperone GroEL
LSA_01280	Genome plasticity	0.6264	0.4783	- 1	transposase insK for insertion sequence element
LSA_12430	Genome plasticity	0.7473	0.6295	- 1	transposase insK for insertion sequence element
LSA_11900	Lipid Metabolism	0.6018	0.4447	- 1	enoyl- ACP reductase

LSA number	COG functional category	1/ 100 Candida Log2 fold changes	1/ 10 Candida Log2 fold changes	Up/down	gene name
LSA_11910	Lipid Metabolism	0.6105	0.3679	- 1	acetyl- CoA carboxylase carboxyl transferase
LSA_11930	Lipid Metabolism	0.6987	0.4047	- 1	Pyruvate carboxylase subunit A
LSA_11940	Lipid Metabolism	0.6528	0.3654	- 1	(3R)- hydroxymyristoyl- ACP dehydratase
LSA_11960	Lipid Metabolism	0.6032	0.4182	- 1	3- oxoacyl- ACP synthase
LSA_11970	Lipid Metabolism	0.602	0.4242	- 1	3- oxoacyl- ACP reductase
LSA_11980	Lipid Metabolism	0.6312	0.4611	- 1	[acyl- carrier- protein] S- malonyltransferase
LSA_11990	Lipid Metabolism	0.6839	0.414	- 1	acyl carrier protein
LSA_12000	Lipid Metabolism	0.5953	0.3808	- 1	3- oxoacyl- ACP synthase
LSA_12010	Lipid Metabolism	0.6831	0.3451	- 1	(3R)- hydroxymyristoyl- ACP dehydratase
LSA_12070	Lipid Metabolism	0.7189	0.6441	- 1	acyl carrier protein
LSA_00280	Membrane Transport	2.0757	3.1814	1	oligopeptide transport ATP- binding protein oppD
LSA_00290	Membrane Transport	2.0714	3.713	1	oligopeptide transport ATP- binding protein oppF
LSA_00300	Membrane Transport	2.8302	5.1172	1	oligopeptide transport system permease oppB
LSA_00310	Membrane Transport	2.0343	3.4509	1	oligopeptide transport system permease oppC
LSA_00320	Membrane Transport	2.0552	3.3517	1	oligopeptide- binding protein oppA
LSA_00340	Membrane Transport	1.2018	1.5698	1	proton/sodium- glutamate symport protein
LSA_00530	Membrane Transport	1.7179	1.0668	0	amino acid permease
LSA_00920	Membrane Transport	1.2173	1.5389	1	phosphonate transport system substrate- binding protein
LSA_01080	Membrane Transport	1.015	0.6181	0	cobalt/nickel transport system permease protein
LSA_02730	Membrane Transport	0.8049	0.6499	- 1	ribose uptake protein rbsU
LSA_04650	Membrane Transport	2.1503	8.1993	1	amino acid permease
LSA_05640	Membrane Transport	0.8318	0.6321	- 1	cobalt import ATP- binding protein cbiO 2
LSA_05650	Membrane Transport	0.8542	0.6078	- 1	cobalt import ATP- binding protein cbiO 1
LSA_05660	Membrane Transport	0.8702	0.5742	- 1	cobalt/nickel transport system permease protein
LSA_01900	Metabolism of Cofactors and Vitamins	0.7607	0.5515	- 1	methylenetetrahydrofolate reductase (NADPH)
LSA_11830	Metabolism of Cofactors and Vitamins	1.1118	1.764	1	phosphomethylpyrimidine kinase
LSA_11890	Metabolism of Cofactors and Vitamins	0.736	0.3517	- 1	BirA family transcriptional regulator, biotin operon repressor
LSA_12060	Metabolism of Cofactors and Vitamins	0.7155	0.5181	- 1	4'- phosphopantetheinyl transferase
LSA_00350	Nucleotide Metabolism	0.9239	0.6226	- 1	2',3'- cyclic- nucleotide 2'- phosphodiesterase
LSA_1p00080	Nucleotide Metabolism	1.5684	1.6539	1	Adenine deaminase 1
LSA_2p00480	Nucleotide Metabolism	0.9384	1.7206	0	cytosine deaminase
LSA_04140	Other	1.32	1.579	1	multidrug resistance protein mdtG
LSA_05020	Other	1.2703	1.7323	1	multidrug resistance ABC transporter ATP- binding
LSA_08950	Other	1.1376	1.7859	1	thioredoxin
LSA_13160	Other	0.5829	0.3363	- 1	Hemolysin- 3
LSA_03230	Protein fate	0.6879	0.5321	- 1	serine/threonine exchanger

LSA number	COG functional category	1/ 100 Candida Log2 fold changes	1/ 10 Candida Log2 fold changes	Up/down	gene name
LSA_06360	Regulatory functions	1.2889	2.0022	1	HTH- type transcriptional regulator YodB
LSA_08880	Regulatory functions	0.7903	0.6429	- 1	catabolite control protein A
LSA_05190	Replication and Repair	1.4872	1.7448	1	UvrABC system protein A
LSA_09040	Replication and Repair	1.6417	0.882	0	DNA polymerase IV
LSA_09710	Replication and Repair	0.9194	1.5252	0	DNA polymerase III subunit delta
LSA_05840	Translation and protein synthesis	0.626	0.8632	0	30S ribosomal protein S14

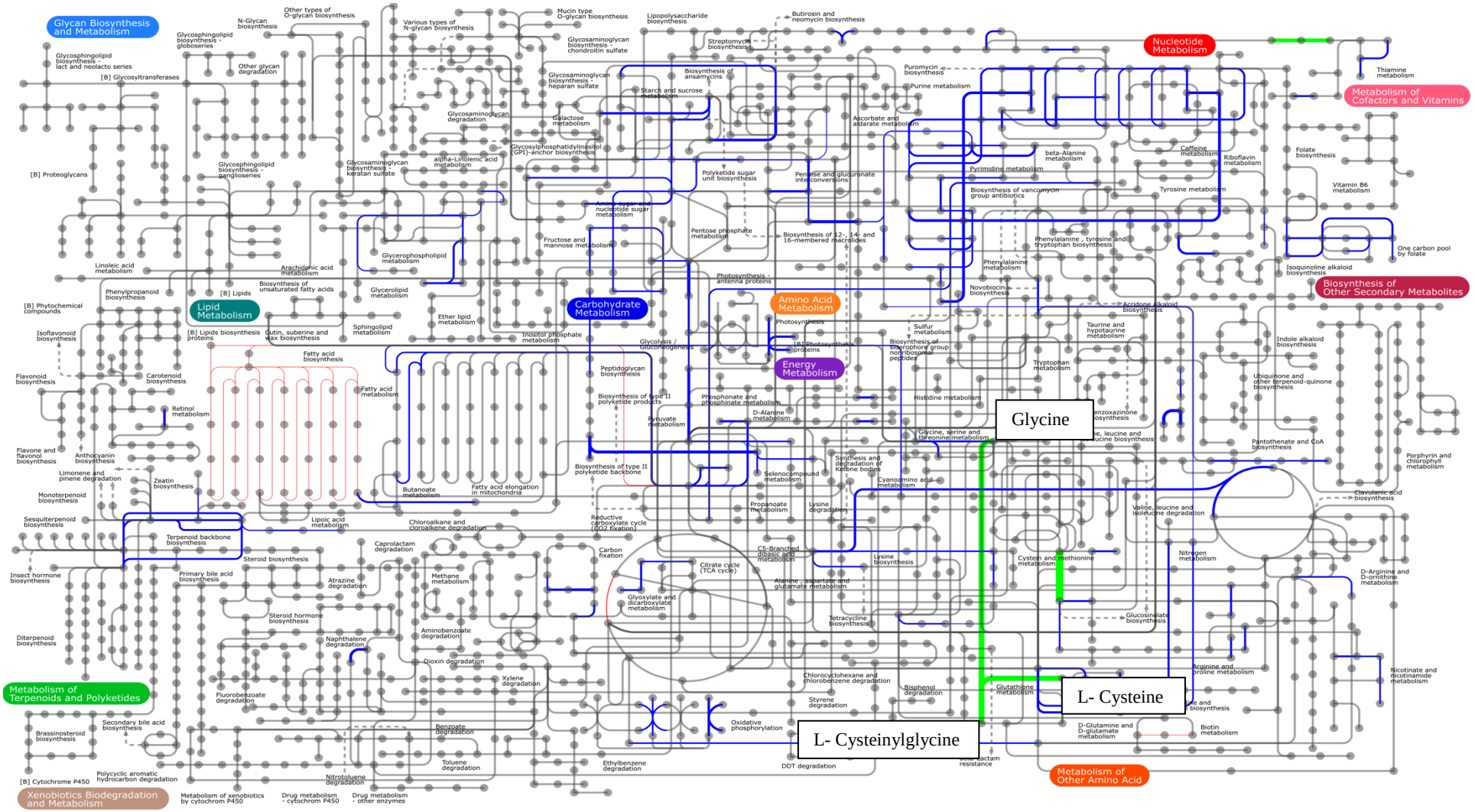


Figure 23: Metabolic pathway visualization of the change in gene expression of *L. sanfranciscensis* TMW 1.1304 incubated with *Candida humilis* TMW 3.191 created with *iPath2* (Letunic, Yamada, Kanehisa, & Bork, 2008), website: http://pathways.embl.de/ Accessed: 03/28/2014. The colors of the pathways indicate the level of gene expression; red mark the underexpressed genes [expression ratios ≤ 0.5]; green are the overexpressed genes [expression ratios ≥ 2] and blue lines show no differential expressed genes [expression ratios > 0.5 < 2). The thickness of the lines represents the level of gene expression ratios, the thicker the colored lines, the higher the corresponding gene expression ratios and vice versa.

4.16 Results of transcriptional analysis using RNA sequencing

4.16.1 RNA quantity and quality check after RNA isolation

The quantity and quality of the extracted RNA samples was again checked using Nanodrop and Bioanalyzer before (Table 29) and after DNAse treatment (Table 30 and Figure 24).

Nanodrop analysis as well as Bioanalyzer results revealed that the sample requirements concerning RNA quality and quantity were sufficient for library construction and sequencing at BGI.

Table 29: RNA quantity and quality measured with Nanodrop® and Bioanalyzer. Visible are the sample names with corresponding RNA quantity and quality values after isolation on two different days. The "+" indicates the treatment with diamide, "- " marks the untreated samples.

Nanodrop®			
sample name	**ng/ µL**	**260/ 280**	**260/ 230**
WT + 11- 041	264,2	2,08	2,5
WT + 28- 033	48,8	2,1	2,61
WT - 28- 034	106,1	2,14	2,19
WT - 11- 042	113,5	2,11	2,19
Δ*tcyB* + 21- 031	130,9	2,09	2,42
Δ*tcyB* +19- 032	154,4	2,1	2,22
Δ*tcyB* - 19- 033	184,3	2,08	2,52
Δ*tcyB* - 21- 034	103,4	2,06	2,45

Bioanalyzer			
sample name	**ng/ µL**	**rRNA ratio**	**RIN**
WT + 11- 041	265	2	10
WT + 28- 033	109	2,1	10
WT - 28- 034	91	2,1	10
WT - 11- 042	97	2,1	10
Δ*tcyB* + 21- 031	138	2,3	10
Δ*tcyB* +19- 032	150	2,4	10
Δ*tcyB* - 19- 033	163	2,3	10
Δ*tcyB* - 21- 034	116	2,3	10

Table 30: RNA quantity and quality measured with Nanodrop® and Bioanalyzer. Visible are the sample names with corresponding RNA quantity and quality values after DNAse treatment and precipitation. The "+" indicates the treatment with diamide, "- " marks the untreated samples.

Nanodrop®			
sample name	**ng/ µL**	**260/ 280**	**260/ 230**
WT + 11- 041	102,7	1,92	2,61
WT + 28- 033	86,8	1,95	2,54
WT - 28- 034	80,6	1,9	2,62
WT - 11- 042	88,4	1,9	2,64
Δ*tcyB* + 21- 031	70,5	1,94	2,48
Δ*tcyB* +19- 032	109,3	1,94	2,54
Δ*tcyB* - 19- 033	85,3	1,92	2,59
Δ*tcyB* - 21- 034	225	2,01	2,5

Bioanalyzer			
sample name	**ng/ µL**	**rRNA ratio**	**RIN**
WT + 11- 041	84	2	9,9
WT + 28- 033	72	2	9,9
WT - 28- 034	67	2	9,9
WT - 11- 042	65	2	9,9
Δ*tcyB* + 21- 031	60	2,3	10
Δ*tcyB* +19- 032	100	2,4	9,9
Δ*tcyB* - 19- 033	70	2,3	10
Δ*tcyB* - 21- 034	225	2,3	9,9

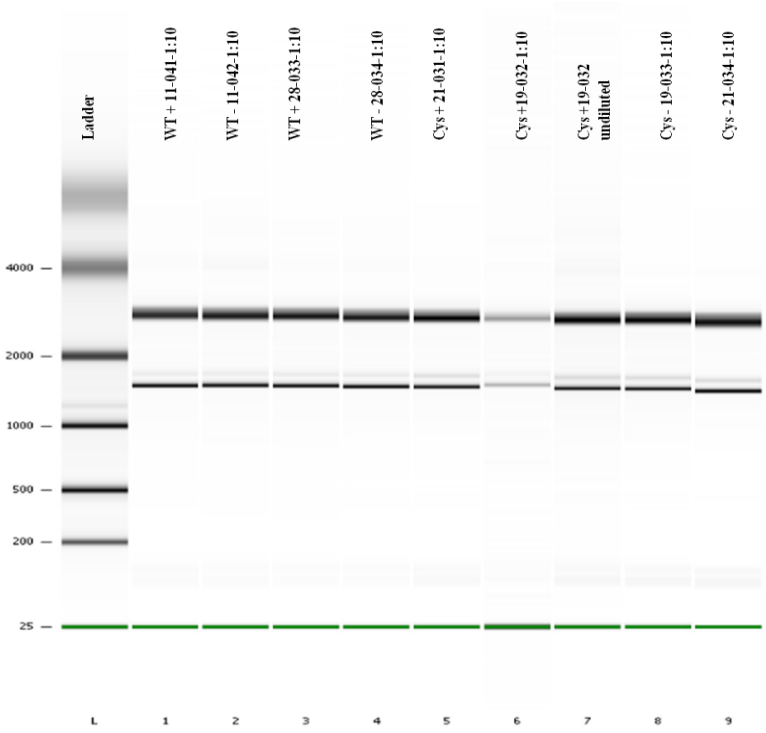


Figure 24: Gel Image (from the Agilent 2100 Bioanalyzer) for total RNA of WT and total RNA of Δ*tcyB* (= Cys) after precipitation. Lanes 1 – 4 show the RNA of the WT of duplicate isolation on two different days (11- 04 and 28- 03), Lanes 5 – 9 show RNA of Δ*tcyB* (= Cys) of duplicate isolation on two different days (19- 03 and 21- 03). Lane L includes the appropriate ladder of the supplier.

4.16.2 FPKM values after quantification

Based on the experimental design, the WT and Δ*tcyB* were treated with diamide, the application of distilled water served as reference (untreated group). Thus, sequencing provided mainly three results of transcriptional responses: 1. Information regarding the treated vs. untreated WT (Table 31), 2. Information about the treated Δ*tcyB* mutant vs. treated WT (Table 32, corresponding bar plot visible in Figure 25) and 3. Information regarding the transcriptional response of the control condition for the untreated WT vs. untreated Δ*tcyB* (Table 33).

Table 31: Significantly differentially expressed genes for WT treated (+) vs. untreated (-). Depicted are the FPKM (Fragments Per Kilobase of transcript per Million mapped reads) values, log2 fold changes and gene descriptions; Up-regulated genes are shaded in grey, downregulated genes are left blank. Isoforms of the genes *amt,ywnH; grpE, hrcA* and *glnH, glnM* are marked in bold.

gene	WT + FPKM 1	WT - FPKM 2	log2 (FPKM 2 / FPKM 1)	Gene description
adh2	3190.67	6685.8	1.0672	aldehyde- alcohol dehydrogenase 2
amt,ywnH	**191.365**	**443.352**	**1.2121**	**ammonia channel**
glnR	296.214	602.319	1.0239	HTH- type transcriptional regulator glnR
glnA	969.914	1856.07	0.9363	glutamine synthetase
groS	621.735	310.704	- 1.0008	molecular chaperone GroES
groL	2350.43	1268.35	- 0.8899	molecular chaperone GroEL
LSA_05000	1897.37	917.327	- 1.0485	pseudogene
glmS	3711.31	1068.47	- 1.7964	glucosamine- fructose- 6- phosphate

gene	WT + FPKM 1	WT - FPKM 2	log2 (FPKM 2 / FPKM 1)	Gene description
				aminotransferase
clpE	1600.8	479.74	- 1.7385	ATP- dependent Clp protease ATP- binding subunit clpE
LSA_04150	588.707	350.071	- 0.7499	hypothetical protein
clpP	8092.43	3903.38	- 1.0519	ATP- dependent Clp protease proteolytic subunit
dnaJ	2412.99	1477.14	- 0.7080	molecular chaperone DnaJ
dnaK	6356.13	4093.29	- 0.6349	molecular chaperone DnaK
grpE,hrcA	**2775.15**	**1610.84**	**- 0.7848**	**protein grpE**
mnmA	3075.42	1789.11	- 0.7815	tRNA- specific 2- thiouridylase mnmA
clpC	1663.07	854.001	- 0.9615	Negative regulator of genetic competence clpC/mecB
ctsR	3916.56	1993.09	- 0.9746	transcriptional regulator ctsR
glnP	270.813	567.37	1.0670	glutamine ABC transporter permease glnP
glnH,glnM	**604.404**	**1415.37**	**1.2276**	**glutamine ABC transporter permease glnM**
glnQ	381.824	791.555	1.0518	glutamine transport ATP- binding protein glnQ

Table 32: Significantly differentially expressed genes for WT treated (+) and Δ*tcyB* treated (+). Depicted are the FPKM (Fragments Per Kilobase of transcript per Million mapped reads) values, log2 fold changes and gene descriptions; Up-regulated genes are shaded in grey, downregulated genes are left blank. Isoforms of the genes *oppD, F* and *glnH/ M* are marked in bold.

gene	WT + FPKM 1	*ΔtcyB* + FPKM 2	log2 (FPKM 2 / FPKM 1)	Gene description
oppD, oppF	**3226.82**	**9794.01**	**1.6018**	**oligopeptide transport ATP- binding protein oppD**
oppB	1740.2	4865.5	1.4833	oligopeptide transport system permease oppB
oppC	1610.97	4592.21	1.5113	oligopeptide transport system permease oppC
oppA	3515.92	7098.13	1.0135	oligopeptide- binding protein oppA
gltT	311.406	509.371	0.7099	proton/sodium- glutamate symport protein
LSA_00850	1063.21	10840.1	3.3499	hypothetical protein
LSA_02330	44.7525	266.704	2.5752	hypothetical protein
spxA	549.505	953.57	0.7952	regulatory protein spx
LSA_03800	652.628	340.516	- 0.9385	hypothetical protein
LSA_03810	445.03	225.341	- 0.9818	hypothetical protein
glnR	302.21	599.889	0.9891	HTH- type transcriptional regulator glnR
glnA	989.525	1721.88	0.7992	glutamine synthetase
LSA_04670	181.256	559.443	1.6260	L- 2- hydroxyisocaproate dehydrogenase
pyrB	412.165	185.494	- 1.1519	aspartate carbamoyltransferase
pyrC	1510.67	690.227	- 1.1300	pseudogene
LSA_05910	440.341	241.258	- 0.8680	pseudogene
yqhL	678.485	1272.45	0.9072	hypothetical protein
LSA_00240	43.453	248.435	2.5153	hypothetical protein
LSA_00590	142.035	708.803	2.3191	hypothetical protein
yxkA	1648.85	2671.09	0.6960	hypothetical protein
nrdH	5508.4	2971.28	- 0.8905	glutaredoxin-like protein nrdH
prsA	4964.1	3008.27	- 0.7226	Foldase protein prsA
dtd	1175.87	2186.74	0.8951	hypothetical protein
relA	260.218	542.357	1.0595	GTP pyrophosphokinase
LSA_08440	132.533	219.523	0.7280	hypothetical protein
LSA_08570	375.05	622.749	0.7316	hypothetical protein

gene	WT + FPKM 1	*ΔtcyB* + FPKM 2	log2 (FPKM 2 / FPKM 1)	Gene description
patA	114.716	457.585	1.9960	aminotransferase A
mnmA	3137.22	5048.64	0.6864	tRNA- specific 2- thiouridylase mnmA
adhA	4132.93	8055.41	0.9628	alcohol dehydrogenase
pyrE	652.102	281.127	- 1.2139	Orotate phosphoribosyltransferase
pyrF	576.405	269.738	- 1.0955	Orotidine 5'- phosphate decarboxylase
glnP	276.29	561.852	1.0240	glutamine ABC transporter permease glnP
glnH, glnM	**616.766**	**1255.13**	**1.0250**	**glutamine ABC transporter permease glnM**
glnQ	389.391	662.733	0.7672	glutamine transport ATP- binding protein glnQ
mnmG	3529.33	1909.38	- 0.8863	tRNA uridine 5- carboxymethylaminomethyl modification enzyme mnmG
mnmE	3122.92	1711.74	- 0.8674	tRNA modification GTPase mnmE

A graphical representation as bar plot of the significantly differentially expressed genes is depicted in Figure 25. The genes with the highest change in gene expression of the treated Δ*tcyB* mutant compared to the treated WT are *oppBCD*, LSA_00850, LSA_02330, LSA_04670, LSA_00240, LSA_00590 and *patA* (marked with black arrows).

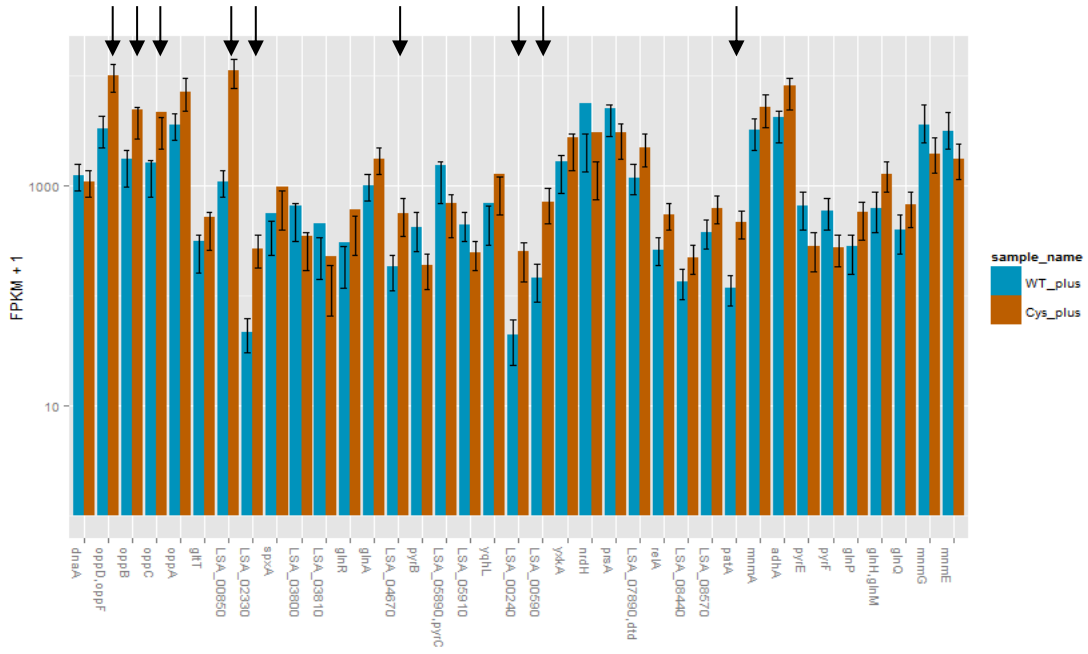


Figure 25: FPKM (Fragments per Kilobase of transcript per Million mapped reads) value bar plot of significant differentially expressed genes of WT treated (WT_plus; blue bars) vs. Δ*tcyB* treated (Cys_plus; orange bars). Visible are the FPKM values including standard deviations plotted against gene names. Black arrows mark the genes with the highest upregulation in the treated Δ*tcyB* mutant.

Using *cummeRbund* package, differential expression of gene isoforms can also be accessed. The different obtained FPKM values of *oppD* (A) and *glnM* (B) are visible in Figure 26. In both images it can be seen that in the treated Δ*tcyB* mutant, the FPKM values for the isoforms TCONS_00000027 and TCONS_00001438 were higher compared to the treated WT.

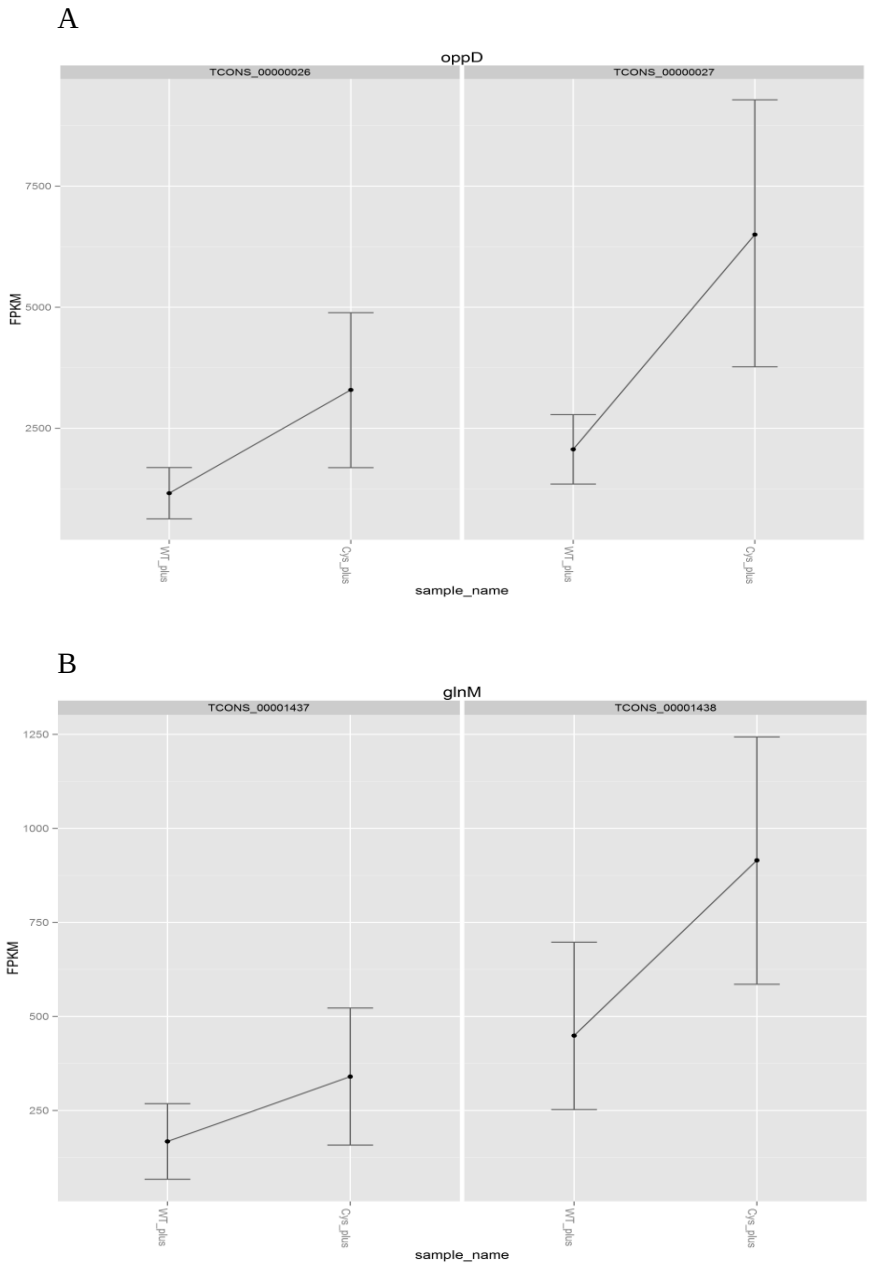


Figure 26: Differential expression of *oppD* (A) and *glnH/M* (B). Isoforms *of oppD and glnH/M* visualized with *cummeRbund* package of *R*. Plotted are the FPKM (Fragments per Kilobase of transcript per Million mapped reads) values against the samples; the abbreviations WT_plus and Cys_plus reflect the wildtype (treated) and Δ*tcyB* (treated). Mean values and standard deviations are displayed of in each case two independent experiments.

Table 33: Significantly differentially expressed genes for WT and Δ*tcyB* untreated (-). Depicted are the FPKM (Fragments Per Kilobase of transcript per Million mapped reads) values, log2 fold changes and gene descriptions. Up-regulated genes are shaded in grey, downregulated genes are left blank.

gene	WT - FPKM 1	Δ*tcyB* - FPKM 2	log2 (FPKM 2 / FPKM 1)	Gene description
LSA_03800	766.695	343.807	- 1.1570	hypothetical protein
LSA_13190	517.663	949.523	0.8752	hypothetical protein
nrdH	4977.65	2282.22	- 1.1250	glutaredoxin-like protein nrdH

4.17 Results of *in-silico* protein prediction analysis

Altogether 287 proteins could be found containing one or several CXXC, CXXS or CXXT motifs. Most of the proteins had molecular weights of 4,500 – 24,000 (30 %) and 25,000 – 49,000 (45.6%) (Figure 27).

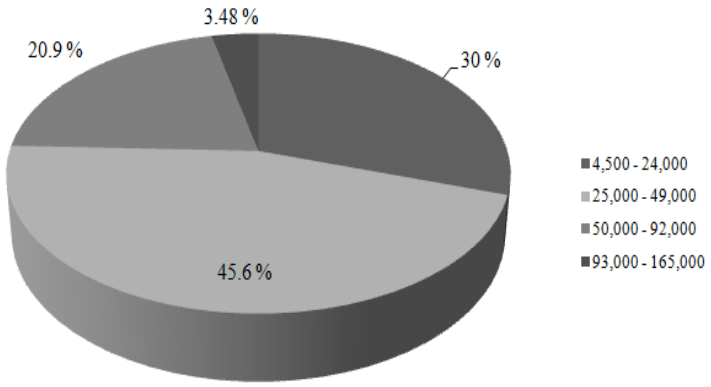


Figure 27: Distribution of *in-silico* predicted proteins with CXXC, CXXS and CXXT motif in the genome of *L. sanfranciscensis* TMW 1.1304 classified in groups with different molecular weights (MW) with corresponding percentages (total of 287 proteins was set as 100 %).

The total number was reduced to 59 selecting only proteins with CXXC (25 proteins), CXXS (21 proteins) and CXXT (13 proteins) motif near the N- terminus of the protein (amino acid position 1 - 40) as known to be specifically redox- active as summarized in Table 34 (Iqbalsyah et al. 2006).

Table 34: Selected overview of predicted proteins with CXXC, CXXS and CXXT motif(s) of *L. sanfranciscensis* TMW 1.1304. Shown are the corresponding gene numbers from NCBI and protein identification numbers, protein annotations, the specific CXX_ signature motifs including amino acid positions, the existence of a thioredoxin-fold (marked in bold, retrieved from PSI- BLAST) and complete length of the proteins.

No	Gene id.	Protein id (NCBI)	Description (annotation)	Signature motif	Location CXXC,S,T (amino acid positions)	Thioredoxin fold (yes/ no)	Length (aa)
1	LSA_04490	YP_004840829.1	DNA repair protein radA-like protein	CSNC CPNC	9- X- X- 12 24- X- X- 27	no	460
2	**LSA_04700**	**YP_004840850.1**	**glutaredoxin-like protein nrdH**	**CIQC**	**21- X- X- 24**	**yes**	**88**
3	**LSA_06080**	**YP_004840964.1**	**thioredoxin-like protein ytpP**	**CPDC**	**39- X- X- 42**	**yes**	**117**
4	**LSA_08950**	**YP_004841227.1**	**thioredoxin**	**CGPC**	**28- X- X- 31**	**yes**	**101**
5	LSA_09640	YP_004841296.1	ATP- dependent Clp protease ATP-binding subunit ClpX	CSFC CNEC	15- X- X- 18 37- X- X- 40	no	433
6	LSA_06150	YP_004840970.1	transcriptional repressor nrdR	CPKC CNNC	3- X- X- 6 31- X- X- 34	no	162
7	LSA_07350	YP_004841081.1	peptide methionine sulfoxide reductase	CFWC	14- X- X- 17	no	176
8	LSA_06030	YP_004840959.1	protein hit	CIFC	8- X- X- 11	no	145
9	LSA_11920	YP_004841502.1	acetyl- CoA carboxylase carboxyl transferase subunit beta	CPIC CPEC	27- X- X- 30 45- X- X- 48	no	278

No	Gene id.	Protein id (NCBI)	Description (annotation)	Signature motif	Location CXXC,S,T (amino acid positions)	Thioredoxin fold (yes/ no)	Length (aa)
10	LSA_06310	YP_004840986.1	50S ribosomal protein L32	CPNC CPSC	30- X- X- 33 43- X- X- 46	no	60
11	LSA_06420	YP_004840997.1	50S ribosomal protein L33	CTEC	9- X- X- 12	no	49
12	LSA_04560	YP_004840836.1	50S ribosomal protein L33	CTIC	10- X- X- 13	no	49
13	LSA_11100	YP_004841430.1	50S ribosomal protein L36	CEQC	11- X- X- 14	no	39
14	LSA_11200	YP_004841440.1	30S ribosomal protein S14	CEHC CRIC	24- X- X- 27 40- X- X- 43	no	61
15	LSA_05840	YP_004840946.1	30S ribosomal protein S14	CERC CRIC	24- X- X- 27 40- X- X- 43	no	61
16	LSA_02490	YP_004840640.1	hypothetical protein	CTRC	16- X- X- 19	no	61
17	**LSA_02610**	**YP_004840651.1**	**hypothetical protein**	**CGPC**	**28- X- X- 31**	**yes**	**111**
18	LSA_03130	YP_004840700.1	hypothetical protein	CKSC	3- X- X- 6	no	539
19	LSA_03750	YP_004840756.1	hypothetical protein	CLGC	34- X- X- 37	no	62
20	LSA_05060	YP_004840879.1	hypothetical protein	CRWC CDKC CVDC CQDC	10- X- X- 13 38- X- X- 41 54- X- X- 57 67- X- X- 70	no	230
21	LSA_06230	YP_004840978.1	hypothetical protein	CIGC CQRC	8- X- X- 11 42- X- X- 45	no	368
22	LSA_11380	YP_004841458.1	hypothetical protein	CTVC	30- X- X- 33 55- X- X- 58	no	216
23	LSA_08850	YP_004841218.1	hypothetical protein	CVIC	6- X- X- 9	no	352
24	**LSA_10270**	**YP_004841357.1**	**hypothetical protein**	**CLKC CKVC**	**12- X- X- 15 31- X- X- 34**	**yes**	**189**
25	LSA_10290	YP_004841359.1	hypothetical protein	CPIC	24- X- X- 27	no	355
1	LSA_04040	YP_004840784.1	Bifunctional protein glmU	CGKS	29- X- X- 32	no	459
2	LSA_09640	YP_004841296.1	Clp protease ATP-binding subunit ClpX	CGKS	18- X- X- 21	no	433
3	LSA_11940	YP_004841504.1	(3R)-hydroxymyristoyl-ACP dehydratase	CLKS	10- X- X- 13	no	153
4	LSA_06360	YP_004840991.1	HTH- type transcriptional regulator YodB	CLCS	14- X- X- 17	no	115
5	LSA_10040	YP_004841335.1	Isoleucyl- tRNA synthetase	CRRS	11- X- X- 14	no	956
6	LSA_13530	YP_004841638.1	membrane protein OxaA	CSNS	23- X- X- 26	no	278
7	LSA_11930	YP_004841503.1	Pyruvate carboxylase subunit A	CIGS	50- X- X- 53	no	456
8	LSA_08520	YP_004841190.1	S- adenosyl-methionine synthetase	CETS	43- X- X- 46	no	396
9	LSA_02130	YP_004840610.1	transposase insF	CYLS	30- X- X- 33	no	178
10	LSA_13500	YP_004841635.1	tRNA uridine 5-carboxymethyl-aminomethyl modification enzyme mnmG	CNPS	20- X- X- 23	no	604
11	LSA_00180	YP_004840441.1	hypothetical protein	CVLS	9- X- X- 12	no	267
12	LSA_01700	YP_004840572.1	hypothetical protein	CLSS	23- X- X- 26	no	322
13	LSA_01720	YP_004840574.1	hypothetical protein	CLQS	21- X- X- 24	no	322

No	Gene id.	Protein id (NCBI)	Description (annotation)	Signature motif	Location CXXC,S,T (amino acid positions)	Thioredoxin fold (yes/ no)	Length (aa)
14	LSA_02490	YP_004840640.1	hypothetical protein	CRHS	5- X- X- 8	no	61
15	LSA_02600	YP_004840650.1	hypothetical protein	CVMS	39- X- X- 42	no	246
16	LSA_03540	YP_004840738.1	hypothetical protein	CITS	8- X- X- 11	no	317
17	LSA_09320	YP_004841264.1	hypothetical protein	CFKS	49- X- X- 52	no	291
18	**LSA_10270**	**YP_004841357.1**	**hypothetical protein**	**CLNS CNSS**	**15- X- X- 18 147- X- X- 150**	**yes**	**189**
19	LSA_2p00130	YP_004841649.1	hypothetical protein	CVCS CSIS	59- X- X- 62 61- X- X- 64	no	236
20	LSA_2p00560	YP_004841685.1	hypothetical protein	CSTS	35- X- X- 38	no	189
21	LSA_1p00140	YP_004841703.1	hypothetical protein	CGVS	26- X- X- 29	no	438
1	LSA_07050	YP_004841055.1	ATP-binding/permease cydC	CLAT	21- X- X- 24	no	580
2	LSA_04510	YP_004840831.1	cysteinyl- tRNA synthetase	CGPT	29- X- X- 32	no	472
3	LSA_07490	YP_004841094.1	dihydrofolate reductase	CGYT	42- X- X- 45	no	163
4	**LSA_04700**	**YP_004840850.1**	**glutaredoxin-like protein nrdH**	**CKMT**	**24- X- X- 27**	**yes**	**88**
5	LSA_10350	YP_004841365.1	glycerol phosphate lipoteichoic acid synthase 1	CLKT	3- X- X- 6	no	663
6	LSA_11550	YP_004841472.1	Lysine- specific permease	CIGT	25- X- X- 28	no	486
7	LSA_04890	YP_004840867.1	O- sialoglycoprotein endopeptidase	CDET	37- X- X- 40	no	366
8	LSA_06150	YP_004840970.1	transcriptional repressor nrdR	CQHT	6- X- X- 9	no	162
9	LSA_10220	YP_004841353.1	tRNA/rRNA methyltransferase	CAGT	23- X- X- 26	no	174
10	LSA_06500	YP_004841005.1	hypothetical protein	CKKT CNWT	7- X- X- 10 85- X- X- 88	no	172
11	LSA_06800	YP_004841035.1	hypothetical protein	CLLT	24- X- X- 27	no	119
12	LSA_01110	YP_004840525.1	hypothetical protein	CELT	14- X- X- 17	no	52
13	LSA_13440	YP_004841629.1	hypothetical protein	CFFT	27- X- X- 30	no	211

Already known genes which participate in oxidation and reduction reactions like glutaredoxin-like protein *nrdH*, thioredoxin-like protein *ytp* and thioredoxin consist of the characteristic thioredoxin-fold (Table 34). The so far uncharacterized hypothetical proteins LSA_02610 and LSA_10270 with a similar size included a thioredoxin-fold at the N- terminus of the protein. Three-dimensional structure analysis of both proteins using 3D-JIGSAW and SWISS-MODEL revealed that the typical thioredoxin-like fold with a three-layer α-β-α sandwich confirmed the previous results (see Appendix A 21 and A 22).

Applying the tight constraints of Gopal et al. (2009), taking proteins with one CXXC motif and with < 130 amino acids resulted in nine proteins for CXXC, two proteins for CXXS and three proteins for

CXXT. The hypothetical protein LSA_10270 would not have been included because it comprised of two CXXC and CXXS motifs and a size of 189 amino acids.

5 DISCUSSION

The present work demonstrates that the cystine transport permease (TcyB) and glutathione peroxidase (Gpo) regulate the redox homeostasis in *L. sanfranciscensis* besides e.g. the NADH oxidase (Nox) and glutathione reductase (GshR).

Nox is needed for a balanced NADH: NAD^+ pool during aerobic conditions. The generation of NAD^+ ensures the reoxidation of compounds via e.g. (lactate or alcohol) dehydrogenase reactions.

In comparison to *nox*, the mode of actions for *tcyB* and *gpo* are different which exert effects even during anaerobic growth conditions. TcyB is essential for maintenance of the intracellular thiol homeostasis through import of cystine with possible reduction to cysteine. Cystine acts not only as nutritional source in *L. sanfranciscensis;* it directly detoxifies H_2O_2 and balances the intracellular thiol pool especially during thiol stress.

Gpo degrades H_2O_2 directly via formation of GSSG and water. The dysfunction of the gene leads to metabolic pathways in which NAD^+ generation is favored. Thus, a possible influence of the NADH: NAD^+ ratio using so far unknown mechanism(s) besides the elimination of H_2O_2 can be suggested.

A detailed description of these findings is explained in the following sections to shed light on the general response to oxidative stress in *L. sanfranciscensis* with special focus on the role of glutathione peroxidase (Gpo) and the cystine transport permease (TcyB).

5.1 Construction of knock-out mutants, distinct growth behavior and quantification of H_2O_2

The construction of deletion mutants for thioredoxin reductase (*trxR*), redox- sensing repressor (*rex*), glutathione peroxidase (*gpo, gpx*) and cystine transport permease (*tcyB*) in *L. sanfranciscensis* DSM20451^T was carried out to further characterize their role in the redox homeostasis of *L. sanfranciscensis*. The insertional inactivation failed for *trxR* and *rex* although transformation procedure; used competent bacteria and chemicals were identical.

As all bacteria exhibit low-molecular-weight thioredoxin reductase, also *L. sanfranciscensis* possesses with CAVC at amino acid positions 133 – 136 the typical active site- containing CXXC motif (Lu & Holmgren 2013). Low-molecular-weight *trxB* are known for their high substrate specificity. It is known from the literature that this enzyme is essential especially under aerobic growth conditions and that reducing agents can improve the survival of deletion mutants (Serata et al. 2012). Despite the addition of DTT to mMRS plates and anaerobic incubation of the plates did not result in any knock-

out transformants for *trxR* so that it can be assumed that an inactivation in *L. sanfranciscensis* is lethal. As no deletion mutants could be obtained with *pME-1*Δ*rex*, an essential role in *L. sanfranciscensis* can also be proposed for *rex*.

For characterization of the mutants, growth in 8 different media was determined. The absence of specific media components which can be involved in redox homeostasis should give first insights in the response of the mutants. It was hypothesized that the deletion of *gpo* and *tcyB* leads to growth limitations during aerobiosis compared to the WT. Growth is impaired if an increased accumulation of ROS occurs if Gpo and TcyB are involved in detoxification mechanisms.

Looking at the minimum µ values for Δ*gpo* and Δ*tcyB* indicated that Δ*gpo* grows worst in mMRS3 (without Mn^{2+}, cysteine and fructose), mMRS4 (without Mn^{2+} and fructose) and mMRS7 (preculture without Mn^{2+}) during aerobic and anaerobic conditions. The Δ*tcyB* mutant in mMRS8 (preculture without cysteine), mMRS4 (without Mn^{2+} and fructose) followed by mMRS3 (without Mn^{2+}, cysteine and fructose) and mMRS7 (preculture without Mn^{2+}). The growth of Δ*gpo* was therefore more affected by Mn^{2+}, whereas growth of Δ*tcyB* was impaired if cysteine and Mn^{2+} were absent.

Cysteine and manganese are already known to be protective for growth and survival of LAB. Mn^{2+} acts as radical scavenger. In the published genome of *L. sanfranciscensis* TMW 1.1304, a *MntH* gene could be identified which codes for a putative manganese transporter which belongs to the family of *Nramp* (natural resistance- associated macrophage protein) proteins. The expression of Mn^{2+} transporters *MntH1* and *MntH2* during aerobiosis could be proven in *L. sanfranciscensis* TMW 1.53 in MRS with added Mn^{2+} (Jänsch et al. 2011). Cysteine with its redox active – SH group has in general distinct roles in protein folding, redox signaling and in antioxidative actions (Hung et al. 2003; Hochgräfe et al. 2007; Netto et al. 2007; Aguirre & Culotta 2012; Horsburgh et al. 2002; Rodrigues et al. 2011). However free intracellular cysteine can also be pro- oxidative in acting as reductant of free iron which leads to DNA damage (Park & ImLay 2003). Regarding the fact that the requirement for iron in LAB is low as already investigated in chemical defined media (Imbert & Blondeau 1998), the protective role of cyst(e)ine in LAB dominates.

The effect of oxygen and resulting ROS formation depended on the media as well. Diminished growth of Δ*gpo* and Δ*tcyB* during aerobic incubation could only be observed in media without Mn^{2+} because µ values were lower compared to the values for anaerobic incubation. This effect was not observable for the WT. The involvement of cystine transport during aerobiosis could be already investigated. A cystine transport permease mutant (*bspA*) of *L. reuteri* BR11 [formerly classified as *L. fermentum* BR11] reached lower OD values than the WT after aerobic incubation (Hung et al. 2003).

An intracellular GSH accumulation and increased Gpo activity during aerobiosis could be seen in *Lactococcus lactis* subsp. *cremoris* SK11 (Fernándes & Steele 1993). It is not known if there exists a similar GSH accumulation mechanism in *L. sanfranciscensis.* An essential role of GSH in *L.*

sanfranciscensis seems likely as the enzymes GshR/ Gor (LSA_2p00270) and Gpo (LSA_09790) are present in the genome. As GSH synthesis is not possible, most likely GSH is transported intracellularly via specific transporters as already known from other bacteria. In *L. sanfranciscensis* TMW 1.1304 the *cydDCA* genes are present, which are organized in an operon and annotated as "ATP- binding/ permease protein". In *E. coli* this ATP- binding cassette- type transporter (*cydDC*), which is also required for cytochrome bd assembly, mediates GSH and to a minimal extent also cysteine transport (Cruz- Ramos et al. 2004; Pittman et al. 2002). The existence of *cydDC* transporter in the genomes of other LAB proposes that GSH is imported and processed (Pophaly et al. 2012b). This assumption is supported by the fact that *L. sanfranciscensis* DSM20451^T tolerance against cold stress induced by freeze- drying, freeze- thawing is increased after previous incubation with GSH (Zhang et al. 2010). The authors demonstrated that GSH prevented oxidation of membrane fatty acids through acting as electron donor for peroxide reduction which is essential in maintaining the structural and functional properties of the membrane (J. Zhang et al. 2012).

Molecular oxygen is converted into superoxide, which cannot be detoxified by SOD in *L. sanfranciscensis*. It lacks this enzyme and also the enzymes catalase, alkyl hydroperoxide reductase (*Ahp*) and NADH peroxidase. The elimination of superoxide and H_2O_2 can only be executed by free available Mn^{2+}. As the used mMRS2 media did not contain Mn^{2+}, especially the mutants Δ*gpo* and Δ*tcyB* were not able to accumulate Mn^{2+} which is known to scavenge ROS (Archibald 1986; Archibald & Fridovich 1981; Archibald & Duong 1984). The mutants reached lower OD values because they had to face increased ROS levels. The phenomenon of Mn^{2+} accumulation is widely distributed in LAB which is proposed to be an efficient strategy as the energy for costly defense regulons can be saved (Horsburgh et al. 2002).

Due to lower growth during aerobic conditions in the absence of Mn^{2+}, it was assumed that an accumulation of H_2O_2 occurs particularly in Δ*tcyB* and Δ*gpo*. It could be evidenced in LAB that H_2O_2 accumulation leads to an early entry into stationary growth phase (Torre & Garel 2000a). Inhibition of enzymes (oxidation of cysteine residues) which mediate glycolysis with concomitant decrease in biomass formation after H_2O_2 treatment could already be shown by Serrano et al. (2007a) and Vido et al. (2005). That *L. sanfranciscensis* is affected by H_2O_2, is supported by the fact that aerobic growth of the WT in the presence of catalase resulted in faster growth (data not shown). The growth arrest after H_2O_2 shock could also be experimentally shown in this work (see results section 4.9). To experimentally validate this theory, accumulated H_2O_2 was quantified in mMRS and mMRS without Mn^{2+} using plates and a quantitative peroxide assay kit.

Significant effects for both mutants could be seen in mMRS without Mn^{2+} and during aerobic incubation. This effect could also be verified using plates with TMB and HRP because the color change for Δ*gpo* and Δ*tcyB* occurred in a shorter time with an increased color intensity compared to the WT.

As the enzyme glutathione peroxidase (Gpo, Gpx) eliminates organic hydroperoxides (ROOH) and H_2O_2 with formation of water and GSSG, the higher concentration of H_2O_2 after gene loss appears intuitive. Consequently, a dysfunction in this enzyme resulted in elevated H_2O_2 levels in *L. sanfranciscensis*. A BLASTP search of the Gpo of *L. sanfranciscensis* TMW 1.1304 indicated a moderate to high (44 – 65 %) homology to glutathione peroxidase of other LAB.

The involvement of the cystine transporter in prevention of H_2O_2 accumulation was already described for a *bspA* mutant of *L. reuteri* BR11 (Hung et al. 2003). It is suggested that H_2O_2 breakdown depends on the level of extracellular cystine which changes between reduced and oxidized forms.

The color change on plates in the absence of Mn^{2+} could hardly be detected. It is known from the literature that Mn^{2+}- containing TMB- peroxidase plates with heterofermentative *Lactobacillus* species increased H_2O_2 formation (Rabe & Hillier 2003). The authors proposed that a stimulation by Mn^{2+} accounts for higher H_2O_2 levels. Nevertheless, Mn^{2+} itself can catalyze H_2O_2 formation as already outlined in the introduction section. In the present work, higher H_2O_2 formation in the presence of Mn^{2+} could also be detected using the Pierce Quantitative Peroxide Assay Kit. Thus, Mn^{2+} increased non- enzymatically the H_2O_2 production besides its role as detoxification agent.

Another observation in the plate assay was that H_2O_2 accumulation by Δ*nox* was not detectable in the plate assay because colonies remained white. Comparing this observation with the results of the Pierce Quantitative Peroxide Assay Kit revealed, that values for both media types (mMRS, mMRS – Mn^{2+}) were above the ones quantified for the WT. Due to the deletion of NADH oxidase-2, increased H_2O_2 accumulation occurred in this strain even in the presence of Mn^{2+} and under anaerobic growth conditions. The generation of H_2O_2 within LAB is carried out by flavoproteins and different enzymes (lactate oxidase, pyruvate oxidase, NADH oxidase-1, L- amino acid oxidase etc.) as already explained in the literature (Marty- Teysset et al. 2000; Anders et al. 1970; Murphy & Condon 1984; Seki et al. 2004b). The *nox*-2 activity in *L. sanfranciscensis* does not account for this effect as water instead of H_2O_2 is produced (Jänsch et al. 2011). The higher H_2O_2 levels in the Δ*nox* mutant propose the participation of other enzymes involved in H_2O_2 accumulation in this strain as mentioned above. Retrieving genome data of *L. sanfranciscensis* TMW 1.1304, no lactate oxidase and no amino acid oxidases for possible H_2O_2 formation could be found. A role of pyruvate oxidase *pox-5* (EC 1.2.3.3/ LSA_00220) in H_2O_2 generation seems likely but a frameshift results in loss of gene function as described earlier (Vogel et al. 2011).

Although differences between the two detection methods existed, observations should not be overrated because the Quantitative Peroxide Assay Kit was more sensitive and reliable compared to TMB and HRP. The insolubility of TMB (solved in DMSO) in aqueous solutions, the different enzyme activity of HRP after incubation of the plates and restrictions in exact quantification are only three reasons which reduce the regular applicability.

5.2 Functional characterization of *tcyB*

The analysis with *Phobius*, which is a transmembrane protein topology and signal peptide predictor revealed, that the cystine transporter TcyB in *L. sanfranciscensis* TMW 1.1304 (LSA_08550) possesses of four predicted transmembrane segments with no signal peptide (Käll et al. 2004). A BLASTP search indicated 74% to 76% homology to cysteine ABC transporter permeases of *L. fructivorans* and *L. florum* which are part of the same phylogenetic group as *L. sanfranciscensis* (Endo et al. 2010; Salvetti et al. 2012). An integral membrane protein encoded by *bspA* locus and which is part of the L- cystine uptake system of *L. reuteri* BR11, shared 55 % homology to TcyB. Sequence homologies of 48 to 69 % to other *Lactobacillus* species proposed that *tcyB* of *L. sanfranciscensis* codes for a cystine/ cysteine ABC transporter permease.

The dysfunction of the cystine transporter in Δ*tcyB* was verified in mMRS and CDM with additional cystine. No growth could be detected in either mMRS (without cysteine) or CDM compared to the WT. The fact that the Δ*tcyB* mutant failed to grow in mMRS – cysteine proposes that the used peptone, yeast and meat extract contained only cystine and no cysteine. This assumption could be confirmed by HPLC measurement in which the cystine concentration ranged between 0.25 - 0.6 mM in used mMRS and cysteine concentrations were to low for quantification. The Δ*tcyB* was still able to transport cysteine which could be investigated in CDM with different concentrations of cysteine (see results section 4.7). There, Δ*tcyB* could even grow little better with 0.285 mM cysteine than the WT which could be an adaptation mechanism in which the cysteine transport in the mutant is increased. Proposed genes which code for proteins involved in cystine transport include LSA_01990, LSA_08550, LSA_08540 and LSA_10490 (Vogel et al. 2011). Taking the results from growth measurements in CDM, *tcyB* seems to be the sole transporter for cystine in *L. sanfranciscensis* DSM 20451^{T}. Generally, the OD values in CDM were very low due to the absence of (oligo) peptides which are preferentially transported and metabolized in *L. sanfranciscensis*. Another reason could be the auxotrophy for twelve amino acids in this strain. Due to this, also the WT depended on cysteine.

5.3 Response of WT, Δ*gpo* and Δ*tcyB* to oxidant treatment

The number of viable cells of stationary and exponentially grown bacteria after H_2O_2 and diamide treatment was tested in mMRS media. As visible in the corresponding results section 4.8, no differences in survival between WT and mutants could be detected. One possible explanation for this finding could be the nutritious incubation media. The used mMRS media comprised of components which can interfere with ROS formation (Mn^{2+}, cysteine, activity of catalase in yeast and meat extract etc.) and therefore indirectly protected the mutants as already mentioned above. The survival and

shock experiment were therefore repeated in CDM which resulted in immediate cell death after application of the agents.

Another explanation for a comparable number of viable cells could be the low concentration (1 mM) of oxidants used. However, it was clearly visible in the results section 4.9, that 1 mM of H_2O_2 is sufficient to cause growth arrest in the WT and mutants during the exponential phase compared to the untreated cells. The reasons were already discussed above. Pre- experiments indicated that much higher concentrations of H_2O_2 led to cell death. Further, it is possible that effects of H_2O_2 and diamide treatment are not visible in the number of viable cells and OD measurements. The bacteria overcome these stresses without changes in cfu. Therefore, extracellular and intracellular thiol groups were determined after H_2O_2 and diamide challenge which will be described in the next passage. The last explanation would be the time of oxidant treatment. It is already known from the literature that the growth phase has enormous influence on bacterial resistance mechanisms (Merrell et al. 2003; Guzzo et al. 2000). As stationary and exponentially grown cultures were tested, an experiment with cells treated with oxidizing and reducing agents at the very beginning of the growth phase was conducted, which is discussed later.

The determination of extra- and intracellular thiol groups should clarify if thiol homeostasis of Δ*gpo* and Δ*tcyB* was affected after H_2O_2 and diamide challenge. In general, thiol groups include all molecules with free –SH group like glutathione, cysteine, possible low-molecular-weight thiols etc.

H_2O_2 treatment increased the extracellular thiol groups in Δ*tcyB* compared to the WT. Simultaneously intracellular thiol groups were significantly lower after H_2O_2 challenge in Δ*tcyB*. The same trend could be observed for the WT although it was not a significant effect. Because intracellular thiol groups of Δ*tcyB* were not significantly lower compared to the WT, action at the outside of the bacterial cell could be one explanation. Due to the deletion of the transporter, cystine remained at the outside of the bacterial cell and interfered with H_2O_2. Due to the missing –SH group, cystine was not captured with the used method using DTNB for quantification, although extracellular and/ or intracellular reduction into cysteine could not be excluded. Nevertheless, effective H_2O_2 elimination by cystine could be already investigated for *L. reuteri* BR 11 (Hung et al. 2003) in which cystine changed between oxidized and reduced forms, depending on the environmental conditions. This finding is supported by the fact that cystine addition prior to H_2O_2 quantification decreased the production of H_2O_2 to undetectable levels (data not shown). Another possibility of unchanged intracellular thiol groups in Δ*tcyB* after H_2O_2 challenge is a more effective import and cleavage of peptides to tightly control the intracellular thiol metabolism.

Another interesting finding was that the extracellular thiol groups of the untreated Δ*gpo* were higher compared to the WT. H_2O_2 led to decreased extracellular thiol levels in this strain. An increase of intracellular thiol groups through thiol import could be excluded as a possible explanation because

intracellular thiol groups were not significantly changed between the treated and untreated group. As Gpo also catalyzes the formation of GSSG and water from GSH and H_2O_2, a deletion leads to higher intracellular levels of GSH, ROOH and H_2O_2. To balance intracellular GSH/ GSSG levels, an export of the increasing thiols (GSH) seems likely. GSH is able to protect membrane SH groups as evidenced before (Hightower et al. 1989). However, a decrease in intracellular thiol levels could not be measured. An explanation could be that H_2O_2 led to a decrease of extracellular bound thiols (GSH) through oxidation of membrane proteins responsible for thiol binding and/ or import or simply oxidation of GSH. The increased extracellular oxidation of GSH into GSSG in the presence of H_2O_2 could already be shown in *E. coli* (Smirnova et al. 2005). As GSSG is not captured by DTNB and GSH is not oxidized in the untreated cells, higher extracellular thiol levels in the control could be measured.

Consequently, untreated bacteria seemed to have more extracellular bound thiols. It is already known that gram- positive aerobic *Firmicutes* tend to exclude cysteine residues from their exported and cytoplasmic proteins (Daniels et al. 2010). It was said that this mechanism could be of advantage because the outer membrane could function as additional barrier, which aids in regulation of cytoplasmic redox homeostasis in extreme environments. Exofacial thiol groups present in membrane proteins and/ or cell wall- bound proteins were also described in *Lactococcus lactis* (Michelon et al. 2010). They are responsible to decrease the redox potential and protect the bacterium during emerging oxidative stress. This fact evidenced that membrane (bound) proteins were so far overlooked to combat damages caused by oxidative stress in *L. sanfranciscensis*. The disturbance in the GSH/ GSSG pool, which was caused by the deletion of *gpo* could probably have opposite effects testing the *gshR* mutant of *L. sanfranciscensis* which accumulates GSSG instead of GSH. A decrease of thiol levels after fermentation of wheat dough with Δ*gshR* compared to the WT could be found which strengthens this assumption (Jänsch et al. 2007).

Looking at thiol groups after diamide treatment showed that the Δ*tcyB* mutant had significantly more extracellular thiol groups than the WT independent of the treatment. The impaired ability to transport cystine increased extracellular thiol levels in mMRS without addition of diamide. A slight trend of Δ*tcyB* having lower intracellular thiol groups as the WT was visible although the effect is not significant. A possibility would be that after 1 hour of diamide challenge the bacteria already adapted through other mechanisms to combat thiol stress efficiently which increased intracellular thiols. The thiol balance is kept tightly balanced to prevent possible enzyme loss, cell arrest and lethality. No significant differences in extra- or intracellular thiol groups could be observed for Δ*gpo*. Thus thiol-oxidizing agent diamide seemed to have no influence on the thiol levels in this mutant. Comparing both oxidant treatments, changes in extra- and intracellular thiol groups for Δ*gpo* and Δ*tcyB* were more pronounced after H_2O_2 exposure.

Although no differences in cfu after oxidant challenge in mMRS could be obtained using bacteria from exponentially and stationary phase, the response of WT and mutants were also tested at the beginning of growth. For that purpose, exposure to H_2O_2, paraquat and diamide in a plate assay was carried out. Using mMRS and mMRS5 plates, Δ*gpo* and Δ*tcyB* showed different growth sensitivities against oxidants in both media types compared to the WT and the control condition. Growth of Δ*gpo* and Δ*tcyB* was affected in the presence of 2.5 and 5 M H_2O_2 in both media types. The presence of H_2O_2 to growing cells of Δ*gpo* and Δ*tcyB* drastically affected their growth. Due to the deletions of *gpo* and *tcyB*, the mutants' resistance against H_2O_2 was strongly impaired because thiol metabolism seems to be affected as discussed above. The findings found for *L. sanfranciscensis* in the present work overlap with previous work in *L. reuteri* BR11, where cystine prevented the formation of H_2O_2 (Hung et al. 2003). The authors proposed that the breakdown of H_2O_2 depended on exogenous cystine. H_2O_2 treatment of *E. coli* cells resulted in induction of the *btuE* gene which codes for a *gpo* (Arenas et al. 2010). A *gpxA* deletion mutant in *Neisseria meningitides* showed increased sensitivity against H_2O_2 treatment whereas aerobic growth was unaffected (Moore 1996).

The Δ*gpo* mutant was more tolerant to high concentrations of paraquat (250 and 500 mM) than the WT. Superoxide stress is better tolerated by Δ*gpo* which is supported by the fact that in the growth tests aerobic incubation in mMRS media increased OD values in a similar way as for the WT. Contrary to these findings are the results in the literature. The deletion of the *gpoA* gene in *Neisseria meningitides* and *Streptococcus pyogenes* increased sensitivity to paraquat (King et al. 2000; Moore 1996). A Δ*gshR* mutant of *L. sanfranciscensis* DSM20451^T showed growth defects in the presence of paraquat in MRS media without added cysteine (Jänsch et al. 2007). One explanation for the obtained results could be the presence of Mn^{2+} in the media or an adaptation reaction which occurred in the preculture where the mutant already encountered increasing concentrations of superoxide radicals and acquired effective antioxidative mechanisms. As an increased resistance against paraquat in mMRS could be observed for Δ*tcyB*, the last explanations could also account for the observation made in Δ*tcyB*.

Significant effects of growth inhibition could be observed for Δ*tcyB* when cells were incubated with paraquat and diamide onto mMRS5 plates compared to the WT. The mutant is not able to overcome superoxide and thiol stress. Increased sensitivity to paraquat was already described in a *bspA* mutant of *L. reuteri* BR11 (Turner et al. 1999). Experiments in *Bacillus subtilis* proposed that diamide causes significant changes in the cysteine biosynthesis pathways with possible S- thiolations in protein thiols (Pöther et al. 2009). Thus, in *L. sanfranciscensis* diamide also affected the thiol metabolism. Cysteine and cystine are interconvertible sulfur- containing amino acids with sites for redox activity and electron transfer, a clear prediction regarding the efficiency of one substance over the other can't be clearly stated.

The mutants Δ*tcyB* and Δ*gpo* had severe problems to grow if the surrounding milieu was oxidative. The tolerance against H_2O_2 (Δ*tcyB* and Δ*gpo*) and diamide (Δ*tcyB*) was diminished at the beginning of the growth phases. Treating the mutants in the exponential phase had no influence on the number of viable cells but changed extracellular and intracellular thiol groups. The question if the bacteria prefer reductive or oxidative growth environments should be answered applying different oxidative and reductive agents.

5.4 Effect of oxidizing and reducing agents on the growth of WT, Δ*gpo* and Δ*tcyB*

Testing the effects of present oxidizing (H_2O_2, diamide) and reducing (GSH, cysteine, DTT) agents, compounds were added to mMRS before inoculation with bacteria followed.

The Δ*tcyB* mutant reached low OD values in the presence of 1 mM diamide, a finding which was not observable for WT and Δ*gpo*. The sensitivity of Δ*tcyB* to diamide as seen in the plate assay could be confirmed in mMRS. The effect of diamide was therefore more pronounced if cells were incubated from the beginning of the growth phase. The thiol- oxidizing agent probably changed the oxidation state of present thiols like GSH, cysteine, coenzyme A etc. (Kosower & Kosower 1995). It seems that especially at the beginning of the growth phase, free SH groups were essential for *L. sanfranciscensis*. Thus, TcyB bound and transported cystine which could be reduced under specific environmental conditions to cysteine. If this mechanism was disturbed, less cysteine became available and increased thiol oxidation through diamide occurred which therefore interfered with the bacterial transport and/ or metabolism. As diamide does not selectively act on cysteine (Kosower & Kosower 1995), overall increase of oxidized thiols (GSSG, protein disulfides etc.) could have significant effects on Δ*tcyB*. A change in extracellular or intracellular thiol groups of Δ*tcyB* after 1 hour of diamide challenge could not be measured. Possible reasons were already discussed in the corresponding chapter. The WT and Δ*gpo* reached similar OD values in the presence of diamide. These results confirmed the findings of the plate sensitivity assay.

The Δ*gpo* and Δ*tcyB* mutants reached higher OD values than the WT after 16 hours in the presence of H_2O_2. This effect was contradictory to the findings already discussed in which a higher sensitivity of Δ*gpo* compared to the WT was reported. As it could be shown in this work that Δ*gpo* and Δ*tcyB* accumulated more H_2O_2 compared to the WT, the mutants possibly adapted because they were used to higher levels of H_2O_2. The adaptation in the preculture probably helped during incubation with manually applied H_2O_2. Nevertheless, OD values remained low already at 1 mM H_2O_2 for all strains. Another possibility lay in the fitness of the preculture of the strains which influenced the follow up culture. If the bacteria experienced stress already in earlier steps (e.g. impaired survival after cryo

culture), their response to oxidants was strongly affected. This is supported by the fact that the OD values for the WT for 5 mM – 100 mM of H_2O_2 looked similar. Due to that, the experiment was repeated for WT and Δ*gpo*. After 5 hours, the Δ*gpo* mutant reached OD values of 0.6 – 0.7 whereas OD values for the WT remained at 0.1 – 0.2. However, as H_2O_2 is a sensitive and active chemical which degrades rapidly, side products resulting from oxidation of media components present in mMRS during the incubation time have to be considered. The resistance against H_2O_2 was further tested in CDM to exclude possible effects of media components. Neither WT nor Δ*gpo* showed any detectable growth, the treatment was lethal for both strains as outlined before.

Results for application of reducing agents (GSH, cysteine, DTT) between WT and mutants looked similar for the concentrations of 1 mM and 5 mM. The WT reached higher OD values with 20 mM GSH but lower values with 20 mM of cysteine compared to Δ*tcyB*. Probably the higher extracellular thiol concentrations for Δ*tcyB* hinder the mutant to import further GSH. The Δ*tcyB* mutant failed in cystine transport but probably acquired mechanisms increasing cysteine transport if cysteine was present in the media. This could be proven using CDM (see results section 4.7) with added cysteine where Δ*tcyB* reached higher OD values compared to the WT. In *L. sanfranciscensis* DSM 20451^T, GSH prevented the loss of activities of enzymes involved in glycolysis which occurred after cold challenge (J. Zhang et al. 2012). GSH had positive effects on the intactness of the membrane with increased fraction of unsaturated fatty acids which protected the bacterium against different cold treatments (Zhang et al. 2010b).

The growth effects of WT and mutants using 100 mM of reducing agents showed also differences mainly between WT and Δ*gpo*, although the effects were not significant due to high standard deviations. The Δ*gpo* mutant reached lower OD values after 16 hours with 100 mM GSH and DTT. A possible explanation would be that deletion of *gpo* increased intracellular GSH which was exported as already stated before (see section 4.10 for quantification of extracellular thiol groups). Therefore, sufficient reducing agents were present, thus the application of additional reductants impaired the growth of Δ*gpo*. It is generally known that *Firmicutes* tolerate high concentrations of reductants (up to 100 mM) whereas growth of other bacteria is already severely affected (Daniels et al. 2010).

5.5 Changes in ORP, pO_2, pH and metabolites during fermentation

Viability and shock tests revealed no differences in the number of viable cells between WT and mutants during the exponential phase. Therefore, strains were fermented in mMRS with recording of ORP, pO_2 and pH values to detect possible differences. Further, the experiment should verify if there exist any connections between the concentration of extracellular thiol groups and redox potential

development as described for *Lactococcus lactis* (Michelon et al. 2010), *E. coli* and *Bacillus subtilis* (Oktyabrskii & Smirnova 2012).

The redox potential curves for WT and Δ*gpo* looked similar. It was assumed that Δ*gpo* reaches lower E_h values compared to the WT due to its ability to increase extracellular thiol levels through GSH export. However, this assumption could not be confirmed as the redox potential between hour 4 and 18 lay above the E_h of the WT. The mutant needed little longer than the WT to reduce present oxygen, which goes along with previous investigations that the strain has additionally to cope with increased ROS (due to higher H_2O_2). The strain was in a more oxidative state than the WT which therefore would explain the more positive ORP values. As these were not significantly different between WT and Δ*gpo*, the importance should not be overrated.

The redox potential of Δ*tcyB* looked different to the E_h of the WT. The Δ*tcyB* mutants needed much longer for reduction of present oxygen in the media. The explanation could be again the delayed growth in mMRS because lower E_h values were firstly reached in the exponential phase of growth. The mutant needed longer to adapt, possibly due to the disturbance of cystine transport which allocates reducing equivalents and minimizes produced H_2O_2.

The redox potential curve of Δ*nox* stayed relatively constant (E_h values > 270 mV) and the pO_2 curve was similar to the one observed for Δ*tcyB*. In the exponential phase of growth (between hour 4 and 10), E_h even rose. Consequently, deletion of NADH oxidase-2 (H_2O producing) resulted not in a decrease of E_h in *L. sanfranciscensis*. When all oxygen was reduced, E_h values dropped again to initial values. These results confirmed the ones obtained from of a Δ*noxE* mutant of *Lactococcus lactis* (Tachon et al. 2010). The authors suggested that elimination of oxygen by *noxE* was necessary to reduce the media especially during the early growth phase and that enzymes of the ETC also contributed slightly to oxygen removal. As *L. sanfranciscensis* has no functional ETC, the oxygen elimination potential in this strain depends possibly on *nox-2* activity (Jänsch et al. 2011). This is an interesting finding because redox potential measurements have not been carried out before in this mutant. Further, the results for Δ*nox* evidenced that the hypothesis of extracellular thiol groups which can also account for the reducing activity could not be confirmed. The increase in thiol groups of Δ*nox* (0.034 mM) did not lead to a fall in E_h values. Similar thiol levels (0.029 – 0.030 mM) could be found for WT, Δ*gpo* and Δ*tcyB* which support this finding.

Significant differences between WT and mutants in produced metabolites could be measured for lactate, ethanol and mannitol. In the first 8 hours, all mutants produced less lactate compared to the WT. The Δ*nox* mutant reached lower lactate concentrations after 8 and 24 hours. As Δ*gpo* and Δ*tcyB* grew slower than the WT, this effect was probably due to delayed growth because after 24 hours the concentrations equal the ones for the WT. As *nox-2* of *L. sanfranciscensis* forms H_2O and NAD^+ from NADH and oxygen, a deletion results in higher $NADH_2$ and lower NAD^+ levels. The formation of

lactate from pyruvate via lactate dehydrogenase generates NAD^+ which is important to balance the NAD^+/ $NADH_2$ pool as visible in Figure 28.

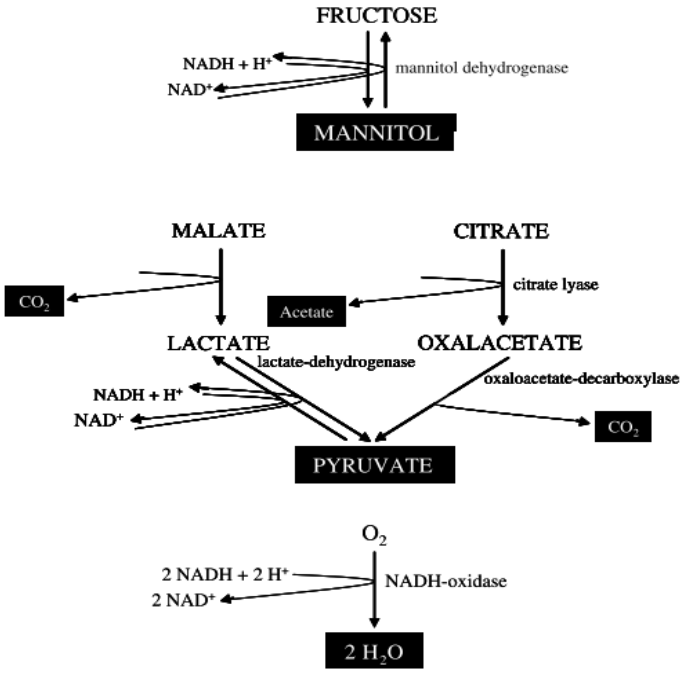


Figure 28: Reactions of potential electron acceptors upon carbohydrate fermentation by *L. sanfranciscensis*, taken from Corsetti & Settanni (2007).

Simultaneously, ethanol concentrations after 8 and 24 hours of Δ*nox* were significantly increased compared to the WT. It is said that *L. sanfranciscensis* directs the carbon flux towards ethanol when reduced pyridine nucleotides were sufficiently regenerated in glycolysis (Jänsch et al. 2011; Knorr und Ehrmann 2001). A significantly higher ethanol concentration after 24 hours fermentation could also be seen for Δ*gpo*. A possibly high and newly regenerated $NADH_2$ pool could explain this. The involvement of Gpo in generation of $NADH_2$ was not described in the literature, although an indirect link to GshR could be found in which $NAD(P)H_2$ served as electron donor to generate GSH.

WT and mutants showed no big differences in maltose, fructose and glucose consumption. The Δ*tcyB* mutant needed longer to make use of maltose, glucose and fructose because of its slower growth. Although it is known that *L. sanfranciscensis* uses fructose exclusively as an external electron acceptor (Jänsch et al. 2011, see also Figure 28), it can be assumed that it can also be converted to generate energy due to discrepancies in conversion rates from fructose to mannitol.

Significant differences could be seen for the quantity of produced mannitol. Exclusively Δ*gpo* and Δ*nox* produced significantly more mannitol compared to the WT. The regeneration of reducing equivalents via NAD(P)H- linked mannitol dehydrogenase and finally ATP generation from acetyl phosphate could represent the reasons for mannitol production (Martinez et al. 1963; Korakli & Vogel 2003).

Probably Δ*gpo* and Δ*nox* produced more mannitol to dispose of a high $NADH_2$ pool which is concomitantly regenerated and which supports also the previous observation of increased ethanol production. External electron acceptors were necessary to balance the overall electron flow. Consequently, both strains benefited somehow from increased mannitol production, firstly in the regeneration of NAD^+ and secondly in increased mannitol formation. Mannitol is known to scavenge hydroxyl radicals in bacteria (Efiuvwevwere et al. 1999), yeasts (Chaturvedi & Bartiss 1997), plants (Shen et al. 1997; Jennings et al. 1998), fungi (Ruijter et al. 2003) and others. Besides its antioxidative capacity this sugar alcohol can serve as carbon source, osmoregulator, as compatible solute etc.. As Δ*gpo* and Δ*nox* produced more H_2O_2 as investigated in this work, the role of mannitol accumulation as antioxidative agent was likely as it already occurred in plants after different stress stimuli (Hare et al. 1998; Chiang et al. 2005) and it played a significant role in the survival of *Lactococcus lactis* after heat stress (Efiuvwevwere et al. 1999).

Similar end concentrations of fructose but higher concentrations of mannitol for Δ*gpo* and Δ*nox* after 24 hours could also lead to the assumption that mannitol was not only produced from fructose. Maybe other sugars from peptone, yeast or meat extract were equally converted. However, the mannitol dehydrogenase (MDH) of *L. sanfranciscensis* is known to take only fructose because no reduction activity for glucose, arabinose, xylose or mannose could be seen (Korakli & Vogel 2003). Another possibility would be that MDH which uses $NADP^+$ or NAD^+ as cofactor has different activities in the presence of increased NAD(P)H levels.

No differences in quantification of amino acids could be seen. As mMRS as complex media included yeast and meat extract with peptides and proteins, conclusions in respect of consumption and synthesis of single amino acids was not possible.

5.6 Distribution of "redox genes" in different LAB

Redox genes which were widely distributed mainly in *L. plantarum, L. brevis* and *L. paracasei* strains included *trxR, gpo,* thioredoxin-like protein (*ytpP*), *npx,* transcriptional regulator *spx* and genes probably involved in reactions of ETC (cytochrome B, nitrate reductase, *nox*).

The ubiquitous nature of thioredoxin reductase and thioredoxin-like proteins explained the high amount of positive PCR signals. Nevertheless, negative results occurred in some *L. paralimentarius* and *L. fermentum* strains.

Only a selection of formerly designed primers could be used for screening purposes. The problems in primer design included the description of gene annotations in the LAB genomes and diminished sequence similarities especially in dehydrogenase genes or small genes involved in actions against oxidative stress. For example, conserved regions in ferredoxin reductase genes were minimal, which resulted in limitations in primer design and many negative results. Even the use of protein sequences for primer design and application of different concentrations of magnesium to increase primer binding could only marginally improve this discrepancy. False- negative results occurred which is supported by a negative signal for the *gpo* gene in *L. sanfranciscensis* TMW 1.53 although the gene is present in the genome. Another problem was the finding of a suitable positive control because primer design was carried out on the basis of DNA or protein sequences of several LAB.

The screening of aroma relevant genes like phenolic acid decarboxylase (*Pdc*), alpha-L-Arabinfuranosidase (*AFN*) and ferulic acid esterase (*Fae*) is not connected to redox homeostasis or antioxidative actions in LAB but shall give additional information about the usability of "redox-active" strains in dough fermentations with possible reactions involved in formation of aroma compounds besides the strains' "redox activity".

Based on the number of positive PCR results, selected strains were classified into five groups. The growth in presence of H_2O_2 and diamide was tested to get information if there are any connections between positive PCR results and tolerance against peroxide and thiol stress. Analyzing the growth curves indicated that results were not clear. Three of five groups (*L. plantarum, L. sanfranciscensis* and *L. fermentum*) showed no differences between presence of "redox genes" and tolerance against applied stressors, whereas PCR results for *L. brevis* and *L. pontis* of a different "antioxidative potential" could be confirmed. PCR Screening has several limitations besides the ones mentioned above. The only presence of "redox" genes does not imply that transcription and translation occurs resulting in "redox- active" proteins. Further, it is not sufficiently known if all of the above chosen "redox genes" used for PCR screening are involved in tolerance against peroxide and thiol stress in the chosen LAB.

In general, the PCR screening was conducted to give a first overview of the distribution of "redox" genes in different LAB. So far, many of the examined strains could be successfully applied in buckwheat sourdoughs including metabolite analysis and thus their practical usability even in gluten-free matrices could be successfully confirmed in our group (Capuani et al. 2014).

5.7 Transcriptional response of *L. sanfranciscensis* TMW 1.1304

Transcriptional analysis has the potential to quantify the mRNA levels and give information, which other genes besides the ones so far described participate in redox reactions. *L. sanfranciscensis* as typical sourdough bacterium is exposed to different environmental stimuli during fermentation. Microarray analysis was used to get insight into the overall transcriptional response of *L. sanfranciscensis* in MRS media in the presence of oxygen, fructose and *Candida humilis*, consequently factors which typically occur in sourdough environments. The dominance of *Candida humilis* in sourdough fermentations besides *L. sanfranciscensis* was described in the literature (Meroth, Walter, et al. 2003; Meroth, Hammes, et al. 2003). Especially the question should be answered if the cultivation with maltose- negative *C. humilis* has positive or negative effects for *L. sanfranciscensis*.

Overall, the majority of genes were not regulated to a statistically significant extent. Significant differentially expressed genes code for small hypothetical proteins and pseudogenes with the highest variation in gene expression (see Appendices A 17 - A 19). Also a high number of differentially expressed genes coding for hypothetical proteins could be found after high pressure treatment of *L. sanfranciscensis* (Pavlovic 2006). This fact exemplified that many genes which code for small proteins or even partially functional proteins played a significant role in this minimalistic bacterium.

As oxygen and fructose both function as electron acceptors, it was expected that the transcriptional response after incubation of *L. sanfranciscensis* was similar. This hypothesis can be partially confirmed. Genes involved in carbohydrate metabolism (glucosamine-fructose-6-phosphate aminotransferase, ribokinase and β-phosphoglucomutase) were downregulated. Due to increased ATP levels, carbohydrate binding (glucosamine-fructose-6-phosphate aminotransferases) and degradation of pentoses (ribokinase) were downregulated. Further amino acid permease and adenine deaminase were upregulated to increase amino acid availability and purine metabolism which are important for cell division. Transcriptional changes of hypothetical proteins in the presence of oxygen and fructose were comparable (see A 17 and A 18) which supports the growth promoting effect of oxygen and fructose through ATP generation (Stolz et al. 1995). Contradictory findings could also be found; the

gene expressions of aminotransferase and homocysteine S- methyltransferase were upregulated in the presence of fructose and downregulated during aerobic incubation.

Distinct differences between the transcriptional response after aerobic incubation and presence of fructose included genes involved in proteolysis, lipid metabolism, nucleotide metabolism and stress response.

Only in the presence of oxygen, genes coding for peptide and amino acid transport (*oppABCDF*, amino acid permease) and protein cleavage (aminopeptidase *pepN*) were overexpressed compared to the control condition. Oligopeptides are internalized and used as carbon and nitrogen source and to replenish damaged cell wall peptides. Microarray data from *L. helveticus* CNRZ32 grown in milk also showed upregulation of genes involved in oligopeptide transport and proteolysis (Smeianov et al. 2007a). The proteolytic system of *L. sanfranciscensis* DSM 20451^T has been partially characterized and it could be shown that expression of peptide transporters was favored during exponential growth in dough (Vermeulen et al. 2005). In *L. sanfranciscensis* it is stated that limited peptide supply in dough rather than increasing nitrogen demand is responsible for this effect (Vermeulen et al. 2005). As oxygen leads to increased ATP generation, faster growth and thereby to a higher biomass, the enhanced need for (oligo) peptides seemed reasonable.

If oxygen was present, genes for fatty acid synthesis (acyl carrier protein, acetyl-coenzyme A carboxylase, (3R)-hydroxymyristoyl-ACP dehydratase) were downregulated. A repression of fatty acid synthesis could also be detected after bile stress in *L. delbrueckii* subsp. *lactis* (Burns et al. 2010), *L. rhamnosus* GG (Koskenniemi et al. 2011) and *L. casei* BL23 (Alcántara & Zúñiga 2012). A change in the composition of bacterial membrane fatty acids resembles a resistance mechanism for many bacteria against diverse stressors as already outlined in the introduction section 1.7.3.

The overexpression of ATP and GMP synthase, adenine deaminase and GMP reductase in *L. sanfranciscensis* indicated that the nucleotide metabolism is activated if oxygen was present. The additional ATP formed via acetate kinase reaction (Knorr et al. 2001; Stolz et al. 1995) was used to form purines which were necessary for further growth of this bacterium.

It appeared that fructose represented less stress for *L. sanfranciscensis* than aerobic incubation because the regulatory protein *spx* and replication and repair enzyme uracil-DNA glycosylase were downregulated. In contrast, aerobic incubation activated *Clp* protease and *uvrABC* system protein A. A similar upregulation of *Clp* proteases could be seen after high pressure treatment in *L. sanfranciscensis* DSM 20451^T (Hörmann et al. 2006). Further, they were induced during heat, salt, oxidative stress, oxygen and glucose limitation in *Bacillus subtilis* (Völker et al. 1994; Gerth et al. 1998). Generally, they were also important for protein quality control in non- stressed cells (Frees et al. 2007). The same role could be observed in *Lactococcus lactis* (Frees & Ingmer 1999; Ingmer et al. 1999). The presence of oxygen accelerated distinct metabolic steps to generate ATP production. However it can also have

side effects because an increase of ROS (e.g. by H_2O_2) occurred which can further lead to damages on proteins, DNA and lipids.

The *uvrABC* complex is involved in DNA repair through excision of a 12 - 13 nucleotide fragment which is replaced by DNA polymerase (Sancar & Rupp 1983). DNA damages which induce *uvrABC* occur after acid stress and stress caused by UV light in *L. helveticus* CNBL1156 (Cappa et al. 2005). A significant higher expression of *uvrABC* could also be measured after H_2O_2 stress in *Bacillus subtilis* (Mostertz, Scharf, Hecker, Homuth 2004). A finding which could not be supported in *E. coli* (ImLay & Linn 1987). In summary, *L. sanfranciscensis* compensated stress caused by oxygen and therefore ROS exposure with increased protein folding/ degradation and DNA repair mechanisms.

The transcriptional response of *L. sanfranciscensis* in co- cultivation with *C. humilis* resembled the response after aerobic incubation in respect of protein, carbohydrate and lipid metabolism. The whole *opp* operon (*oppABCDF*), the aminopeptidase *pepN*, aminotransferases A and amino acid permease were upregulated whereas genes for carbohydrate (β-phosphoglucomutase, gluconokinase, ribokinase) and lipid metabolism (enoyl-ACP reductase, acetyl-CoA carboxylase carboxyl transferase, S-malonyltransferase) were downregulated.

Remarkably, the number of genes involved in stress responses was increased during co- cultivation with *C. humilis* compared to aerobic incubation. Besides upregulation of *Clp* protease and *uvrABC* as observed in the presence of oxygen, gene expressions of thioredoxin, universal stress protein (*uspA*), molecular chaperone *GroES*, multidrug resistance protein and ABC transporter and peptide methionine sulfoxide reductase (*msrA*) were increased.

Thioredoxins are small proteins which become oxidized during oxidative stress as already outlined in the introduction section 1.3.2.2. The essentiality of thioredoxin- thioredoxin reductase system as major thiol/disulfide redox system in LAB was described in the literature (Serata et al. 2012). In *Bacillus subtilis*, thioredoxins act as thiol- disulfide oxidoreductases on the outer side of the cytoplasmic membrane (Möller & Hederstedt 2008). Expression of thioredoxin was induced after H_2O_2 and diamide challenge (Jobin et al. 1999; Uziel et al. 2004) and sensitivity against both oxidants was increased in *trxA* mutants of *Rhodobacter sphaeroides* (Li et al. 2003). It seemed that the co-cultivation with *C. humilis* caused partially a stress response in *L. sanfranciscensis* which is comparable to peroxide and thiol stress known from other bacteria.

It is known from previous work that upregulation of *uspA* occurred during different stress stimuli. The *uspA* was upregulated during p- coumaric acid and peroxide stress in *L. plantarum* (Reverón et al. 2012; Stevens 2008). A significant higher expression of *uspA* after cold challenge supplemented with GSH was also observed in *L. sanfranciscensis* DSM 20451^T (J. Zhang et al. 2012). No overexpression of *uspA* could be seen for *L. helveticus* CNRZ32 during normal growth conditions (Smeianov et al.

2007b) which supports the fact that *L. sanfranciscensis* encountered stress in the presence of *C. humilis*.

The overexpression of the molecular chaperone *groES* indicated that this bacterium was damaged by false protein folding and/ or protein accumulation and degradation. The so- called typical heat shock genes are known to be upregulated during high temperature (Li et al. 2011; Walker et al. 1999). An overproduction of *groESL* in *L. paracasei* NFBC 338 led to improved cell integrity after spray- and freeze- drying (Corcoran et al. 2006). An involvement during acid stress and subsequent adaptation was discussed for *L. bulgaricus* (Fernandez et al. 2008). The co- cultivation probably changed intracellular proteins in *L. sanfranciscensis* with possible effects on cell integrity.
The multidrug resistance systems (MDR) prevented the import of harmful compounds like antibiotics which are necessary for bacterial survival (Konings et al. 1997). The upregulation of multidrug resistance protein and multidrug resistance ABC transporter proposed that *C. humilis* produced compounds which were toxic for *L. sanfranciscensis* because proteins for active export of these compounds were activated.

Peptide methionine sulfoxide reductases (*msrA, msrB*) reduce oxidized methionine residues back into methionine as already described in introduction section 1.7.1. Increased expression could be detected after peroxide and superoxide stress in *Bacillus subtilis* (Mostertz, Scharf, Hecker, Homuth 2004). Two mutants (Δ*msrA* and Δ*msrB*) of *Enterococcus faecalis* showed elevated sensitivity to H_2O_2 (Zhao et al. 2010). These facts evidence that co- cultivation with *C. humilis* led somehow to increased oxidations of methionine. One possibility was the increased production of oxidants (H_2O_2, superoxide etc.) in *C. humilis* which can permeate freely through membranes and damage intracellular proteins of *L. sanfranciscensis*. As this bacterium prefers a reductive environment during growth, the co-cultivation probably oxidizes the surroundings with the described effect of methionine oxidation.

Beside the involvement of oxidative stress genes, one lactate dehydrogenase (LDH) was over- , the other underexpressed. The gene L-2-hydroxyisocaproate dehydrogenase (Malate DH, LSA_04670) was overexpressed as well. The formation of lactate or malate from pyruvate is regulated depending on the intracellular NAD^+/ NADH ratio.

The visualization with *iPath2* revealed that besides the downregulation of lipid metabolism, the conversion and formation of the glutathione precursor peptide cysteine-glycine was upregulated. The synthesis of glutathione is unfeasible due to the absence of enzymes like γ-glutamyl-cysteine synthetase (GshA), glutathione synthetase (GshB) or glutathione biosynthesis bifunctional fusion gene (GshA/B/ GshF) and proposes therefore a role of the mentioned dipeptide. As the reactive thiol group of cysteine is still present, a role in thiol redox homeostasis in *L. sanfranciscensis* could not be excluded. The presence and "antioxidative" potential against ROS formation of low-molecular-weight thiols (LMWT) like bacillthiol (BSH) in *Bacillus subtilis* (Gaballa et al. 2010; Zuber 2009) or ɤ-glutamylcysteine (ɤ-GC) in *Leuconostoc* spp. (Kim et al. 2008) could already be evidenced. A possible

role of redox- active dipeptides is confirmed by the fact that several low-molecular-weight thiol compounds were detected in bacteria lacking GSH synthesis (Gaballa et al. 2010; Kim et al. 2008; Liebert et al. 2006).

Altogether, aerobic incubation represented a similar stimulus as fructose addition. The co- cultivation was not beneficial for *L. sanfranciscensis* as many genes involved in stress response were upregulated. Probably, the bacterium faced a more oxidized environment when incubated with the yeast. As the transcriptional response of the yeast could not be analyzed, the question could not be answered if it was somehow stimulated or even negatively affected by the bacterium.

Despite several advantages in the use of microarray analysis, some limitations of the experimental design have to be taken into account. The sample collection was carried out on one time point during the exponential phase. Therefore, conclusions have to be drawn carefully because changes in gene expressions are only specific for this particular growth phase. What happens during other growth phases lies beyond the informational value of this work. Lastly, as the experiment was conducted only one time, the informational value concerning the inter- assay reproducibility is limited. However, the primary aim of this experiment was to get an overview about the overall transcriptional response of *L. sanfranciscensis* during stimuli typically present in sourdough- like fermentations.

5.8 Transcriptional response of *L. sanfranciscensis* WT and Δ*tcyB* after diamide treatment

As diamide had several effects on Δ*tcyB* as observed in this work (increased sensitivity on plate assay, growth disturbances), RNA sequencing should shed light on changes in the intracellular thiol metabolism and possible adaptation reactions which can hardly be seen using other experimental methods. For this purpose, WT and Δ*tcyB* were treated with diamide and therefore gene expression changes could be compared (1) from the treated vs. untreated wildtype and (2) the stress responses of treated wildtype vs. treated Δ*tcyB* mutant. The experiment was conducted with 1 mM of diamide which seemed reasonable because growth effects on *E. coli* could be seen using 2–3 mM diamide (Hondorp & Matthews 2004).

5.8.1 Thiol stress response of the WT

The majority of upregulated genes resembled the bacterial response after thiol stress induction. Higher FPKM values could be detected for molecular chaperones *dnaJK*, the heat shock protein *grpE* and the heat shock response transcriptional regulator *hrcA*; the chaperones *groESL*, the ATP- dependent *clp* proteases *clpEPC* and the transcriptional regulator *ctsR*. In *E. coli*, the chaperones *dnaK* and *dnaJ* degrade misfolded proteins (Kandror et al. 1994). In other bacteria the proteins were induced during heat stress (Han et al. 2005; Homuth et al. 2000; Koch et al. 1998; Okamoto- Kainuma et al. 2004). High pressure treatment of *L. sanfranciscensis* DSM 20451^{T} caused overexpression of proteins with sequence homologies to heat shock proteins *dnaK*, *groEL* and *clp* proteases (Hörmann et al. 2006) as already mentioned above. The operon structure *hrcA-grpE-dnaK-dnaJ* could be described in *Bacillus subtilis* (A. Schulz 1996), *Enterococcus faecalis* (Laport et al. 2004), *Streptococcus mutans* (Lemos et al. 2001) and others. This operon was activated after heat, salt and ethanol stress in *L. sakei* (Schmidt et al. 1999).

The role of *Clp* proteases in the presence of different stressors and in general protein quality control was already described above. *Clp* expression is controlled by the regulator *ctsR* through specific binding to the promoter regions of *clpC, E* and *P* as known for *Bacillus subtilis* after heat stress and from several other gram- positive bacteria (Derré et al. 1999). In *Oenococcus oeni dnaK* and *groESL* are controlled by *ctsR* (Grandvalet et al. 2005), in *Streptococcus salivarius* the control of *clp* and chaperone expression was mediated through *ctsR* and *hrcA* (Chastanet & Msadek 2003). Previous findings indicated that besides *hrcA*, two *ctsR* repressor homologues exist in the genome of *L. sanfranciscensis* which also seems to be important during sublethal high pressure treatment (Pavlovic 2006).

Thiol stress in *L. sanfranciscensis* induced stress- inducible proteins which overlapped with stress responses after heat and/ or cold treatment. A tailor made stress response does not seem to exist in *L. sanfranciscensis*, a finding which could already be observed after high pressure treatment (Pavlovic et al. 2005).

Looking on amino acid metabolism, glutamine transport genes (*glnHM, P, Q*), the glutamine synthesis (*glnA*) gene and the transcriptional regulator (*glnR*) in *L. sanfranciscensis* were repressed. This unexpected mechanism was quiet surprising because a connection between thiol stress and glutamine metabolism seemed far from obvious. Glutamine represents an important nitrogen source. The function of *glnR* depends on *glnA* as investigated for other bacteria (Kormelink et al. 2012). *GlnR* regulates transcription of genes which are connected to glutamine and glutamate synthesis as well as glutamine uptake. The activity depends on intracellular glutamate, glutamine and ammonium levels (Kloosterman et al. 2006). It could be shown that *glnR* regulated ammonium transport in *L. plantarum*, *Lactococcus lactis* and others (Doroshchuk et al. 2006; Fink et al. 2002; Kormelink et al. 2012). Glutamine formation from glutamate and ammonium was carried out by glutamine synthetase (*glnA*), the equal repression of glutamine and ammonia import lowered therefore intracellular glutamine levels in *L. sanfranciscensis*.

Further, overexpression of *glmS* (glucosamine-fructose-6-phosphate aminotransferase) occurred in the WT. The formation of D-glucosamine-6-phosphate and L-glutamate from D-fructose-6-phosphate and L-glutamine is catalyzed by this enzyme. However, as the reaction goes in both directions, the generation of D-fructose-6-phosphate and L-glutamine was favored because feedback regulation repressed genes involved in glutamine synthesis and uptake as mentioned before. Thiol stress downregulated *adh2* which reflected a physiological mechanism in which the bacteria balanced its NAD^+/ NADH pool.

5.8.2 Thiol stress response of Δ*tcyB*

Looking at the transcriptomic data for Δ*tcyB*, a slight upregulation of *spxA* could be observed. The *spx* protein as explained in the introduction section 1.5.3, is a member of the arsenate reductase family, which is known to be activated after different stress stimuli (low pH, presence of antibiotics, high temperatures, detergents and ROS). It leads to repression or activation of transcription of genes involved in diverse processes in several low- GC gram- positive bacteria (Nakano et al. 2003; Kajfasz et al. 2012; Smith et al. 2010). The deletion of *spx* increased sensitivity during low pH, high temperature and in the presence of oxygen, H_2O_2 and diamide (Kajfasz et al. 2012). Similar observations could be seen in *Bacillus subtilis*, *Staphylococcus aureus* and *Streptococcus mutans* in which *spx* influenced transcription of genes, which were associated with oxidative stress (Kajfasz et al. 2010; Pamp et al. 2006; Zuber 2009). It is known from *Bacillus subtilis* that *spx* controls cysteine

biosynthesis genes, a finding which could also be possible in *L. sanfranciscensis* regarding the fact that no upregulation of *spx* could be detected for the treated WT. The WT tolerated thiol stress much better compared to Δ*tcyB*. Diamide disturbed the intracellular cysteine and methionine pool as investigated in *Bacillus subtilis* and *Staphylococcus aureus* (Pöther et al. 2009) by leading to posttranslational protein modifications like protein S- cysteinylation or oxidation of protein thiols (Hochgräfe et al. 2007). The redox- active thiol groups from cysteine or from converted cystine are used to reduce the increasing number of disulfide bonds. Due to the absence of a cystathionine-ɤ-lyase in the genome of *L. sanfranciscensis* TMW 1.1304, another mechanism seem to exist for the liberation of cysteine than that one described for *L. reuteri* BR11 (Hung et al. 2003).

The expression of *nrdH* (Glutaredoxin-like protein) was significantly higher in the WT than in Δ*tcyB* independent of the diamide treatment (see Table 32 vs. Table 33 in results section). The mutation in the cystine transporter caused expressional changes in *nrdH* which are not influenced by diamide treatment. An increased sensitivity of Δ*nrdH* mutant of *Staphylococcus aureus* against H_2O_2 and diamide stress could not be observed which excluded the possibility of participation in actions against oxidative stress (Rabinovitch et al. 2010). The present work supported the finding that the *nrdH* protein seems to be connected with cystine transport and/ or metabolism in *L. sanfranciscensis*. As efficient reductant, *nrdH* proteins act on disulfide bonds of small molecules and serve as electron donors with high specificity for class Ib ribonucleotide reductases (RNR) (Rabinovitch et al. 2010). They can be found in bacteria lacking GSH, thus an effective reduction of low-molecular-weight thiols through *nrdH* in *L. sanfranciscensis* seems reasonable.

Interestingly, the whole *opp* operon (*oppD*, *oppF*, *oppB*, *oppC* and *oppA*) was higher expressed in Δ*tcyB* stressed with diamide. Due to a decrease in cystine transport, the bacterium adapted by increasing the oligopeptide transport to release cystine from oligopeptides. The aminopeptidase *pepN* is located right after the *oppA* gene and it could already be evidenced that both are part of the same operon (Vermeulen et al. 2005). During thiol stress the Δ*tcyB* mutant required more oligopeptides than the WT. Thus, cystine itself participated in balancing the thiol state in this bacterium. The increase of extracellular thiol levels of Δ*tcyB* vs. WT as evidenced and discussed above support this assumption. The in- time cleavage of imported oligopeptides into amino acids seems therefore highly probable and explains the unchanged concentration of intracellular thiol groups after diamide challenge discussed in section 5.3.

The higher expression of *oppD*, *oppF*, *oppB*, *oppC*, *oppA* and *pepN* in *L. sanfranciscensis* could already be seen during aerobic incubation and in co- cultivation with *C. humilis* (chapter 5.7). Thus, it seems that in the presence of different stressors (oxygen, presence of *C. humilis*, thiol stress), the strain expressed its oligopeptide transporters and aminopeptidase to increase tolerance against damages. Peptidases are used as carbon and nitrogen sources but are also used to recycle damaged peptides in the bacterial cell wall. In *L. sanfranciscensis*, the expression was favored during exponential growth

and when peptide supply was limited (Vermeulen et al. 2005). Nevertheless, these effects would have also been occurred in the control conditions in the WT which supports the hypothesis that increasing oligopeptide transport and cleavage has other effects in this bacterium besides replenishment of peptides and/ or amino acids. In the literature, chaperone-like functions were described in which oligopeptide transporters can assist in protein (re)folding and renaturation (Richarme & Caldas 1997). An involvement in signaling was also described as so- called peptide pheromones which were imported by oligopeptide transporters, interacted with intracellular receptors and changed gene expressions especially in gram- positive bacteria (Azcarate- peril et al. 2005; Lazazzera 2001). Thus, increased expression of oligopeptide transport could be also a stress induced mechanism. This finding could be seen for *L. bulgaricus* in which oligopeptide transport and peptidase expression were decreased after acid adaption (Fernandez et al. 2008) but also in the present work for *L. sanfranciscensis* in which *opp* was only significantly higher expressed after different challenges.

A high increase in gene expression could be measured for the hypothetical protein LSA_00850 in the Δ*tcyB* compared to the WT. Using BLASTP an 85 % homology of an uncharacterized lipoprotein which probably transports methionine in *L. florum* could be found. The close phylogenetic relationship of *L. florum* and *L. sanfranciscensis* could already be evidenced (Endo et al. 2010). Thiol stress caused depletion in cystine and methionine levels in Δ*tcyB*. The significant higher expression of *relA* supports the result that starvation of intracellular amino acid occurred. An accumulation of guanosine-3'-diphosphate-5'-triphosphate [(p)ppGpp] after *relA* activation which is known as the alarmon of the stringent response could be seen after disulfide stress and amino acid deprivation (Pöther et al. 2009). The importance of *relA* in balancing growth, control of catabolic pathways and in survival was described for *Streptococcus mutans* (Nascimento et al. 2008). An accumulation of (p)ppGpp in *Enterococcus faecalis* interfered with amino acid synthesis, stress survival and tolerance against antibiotics (Abranches et al. 2009).

Diamide treatment and oxidative stress in general led to inactivation of a cobalamin- independent methionine synthase (*MetE*) in *E. coli* through oxidation of cysteine 645 (Hondorp & Matthews 2009; Hondorp & Matthews 2004). The changes in enzyme activity with a decrease in methionine synthesis led to lower intracellular methionine concentration. Diamide treatment caused S- thiolations as known for *Staphylococcus aureus* and *Bacillus subtilis* (Pöther et al. 2009). Signal transduction and enzyme activities were influenced by oxidation of thiol groups in proteins or low-molecular-weight thiols as stated for *E. coli*. A damage of free or protein- bound methionine was supported by the fact that expression of *msrB* (methionine sulfoxide reductase B) was significantly higher in treated vs. untreated Δ*tcyB* mutants (see A 20 in Appendix section). Oxidized methionine can be reduced back by *msrA* and *msrB*. An increase in *msrB* expression could not be detected for the WT. A similar differential expression of *msrA* could not be seen for the WT or Δ*tcyB* independent of the conditions. It could already be tested that *L. sanfranciscensis* TMW 1.1304 is auxotroph for cysteine and

methionine in chemical defined media (data not shown). Thus, the higher need for methionine is rather carried out via methionine transport and increase in methionine reduction through *msrB* than *de novo* methionine synthesis.

The proteins LSA_03800 and LSA_03810 have sequence homologies (52 to 79%) to branched chain amino acid (BCAA) transporters of other *Lactobacillus* spp. as retrieved from BLASTP search. Aligning the nucleotide sequence of LSA_03800 of *L. sanfranciscensis* with an *azlC* protein (branched chain amino acid transporter, Accession no. AJ937238) of *L. reuteri* LTH5531 indicated 72 % homology. Thiol stress caused by diamide resulted in a decrease of gene expression values in Δ*tcyB.* An upregulation of genes which participated in BCAA breakdown in *L. sanfranciscensis* LSCE1 could be detected after acid stress (Serrazanetti et al. 2011). The cellular redox homeostasis is tightly controlled; decreasing the concentration of BCAAs similarly to glutamine concentration could be a possible mechanism. It is described for other gram- positive bacteria that changes in BCAA biosynthesis pathways occurred after diamide challenge with accompanied alleviation of valine and isoleucine concentrations (Pöther et al. 2009). The activation of the alarmon of the stringent response through (p)ppGpp accumulation was already mentioned above. It could be seen that a (p)ppGpp deletion mutant (Δ*relAPQ* strain) of *Streptococcus mutans* failed to grow in media without leucine and valine (Lemos et al. 2007). Besides a decrease of intracellular methionine levels, reduced BCAA levels caused *relA* activation. Consequently, a higher (p)ppGpp pool led to decreased expression of BCAA transporters. It was investigated for *Bacillus subtilis* that BCAAs activated *codY*, which on the other hand repressed genes involved in BCAA synthesis (Molle et al. 2003). However, this mode of action can not be transferred to *L. sanfranciscensis* because no *codY* homologue could be found in the genome of *L. sanfranciscensis* TMW 1.1304.

Diamide treatment increased expression of alcohol dehydrogenase *adhA* in Δ*tcyB.* In the presence of external electron acceptors like fructose, citrate or oxygen, acetate formation occurred in heterofermentative LAB. As the growth media contained fructose, the formation of ethanol seems implausible because normally acetate and ATP formation via acetate kinase reaction is favored. The oxidation of ethanol generated acetaldehyde and NADH. Probably the demand of reducing equivalents like NADH of the Δ*tcyB* mutant was increased during thiol stress; thus, the cell boosted its ethanol formation via activation of *adhA.*

The genes carbamoylphosphate synthase (*pyrA*), aspartate transcarbamylase (*pyrB*), dihydroorotase (*pyrC*), dihydroorotate oxidase (*pyrD*), orotate phosphoribosyltransferase (*pyrE*) and orotidine monophosphate decarboxylase (*pyrF*) take part in de novo biosynthesis of pyrimidine nucleotides. A decrease in gene expression could be measured for *pyrB, C, E* and *F* in Δ*tcyB* after diamide challenge compared to the WT. In *L. sanfranciscensis* the genes *pyrDA, B* and *C* seem to be part of one operon whereas *pyrE* and *F* are located in another area on the chromosome. As the bacterium faced increased damages caused by thiol stress, survival had supreme priority. Therefore, pyrimidine synthesis as

energy costly process was shut down. In *L. plantarum* the *pyr* operon is regulated by transcription attenuation (Elagöz et al. 1996). It is proposed that initiation of transcription depends on the presence of uracil in the growth media (Nicoloff et al. 2005). The gene responsible for uracil transport in *L. sanfranciscensis* is called uracil permease and is not part of the *pyr* operon. It could be already investigated in CDM that *L. sanfranciscensis* is unable to grow without additional uracil, pyrimidine and purine bases (data not shown). The RNA binding regulator *pyrR*, which is part of one operon in *Bacillus subtilis* and *L. plantarum*, repressed the expression of *pyr* genes in uracil- free media (Arsène-Ploetze et al. 2006). Uracil has probably other functions in *L. sanfranciscensis* besides repressing pyrimidine synthesis because in contrast to *Bacillus subtilis* and *L. plantarum*, the *pyrR* regulator could not be found within the *pyr* operon.

One of several other advantages of RNA- sequencing was the analysis of regulations of gene isoforms. Significantly differentially expressed isoforms could be extracted from the sequencing files. Exemplarily the isoforms of *oppD*/ *oppF* and *glnH*/ *glnM* were visualized in this work. Interestingly, both gene isoforms of WT and Δ*tcyB* responded differently after diamide treatment. The isoforms *oppD* and *glnM* showed significant higher FPKM values in the Δ*tcyB* mutant compared to the WT which indicated that the deletion of the cystine transporter even influenced their gene expression. The importance of present isoforms expands the functional properties of enzymes especially in a bacterium with a minimalistic genome. However, general information about the role of specific isoforms in LAB is lacking.

Diamide led to a transcriptional response in *L. sanfranciscensis* which is known from other bacteria to occur after heat, cold or high pressure challenge. The thiol metabolism seemed to be not affected as long as thiol homeostasis can be kept balanced through extracellular thiol binding and import. Changes in the intracellular thiol homeostasis in *L. sanfranciscensis* appeared after deletion of the cystine transporter. The typical adaptation reaction included increased import of oligopeptides which are immediately cleaved into amino acids, which can partially compensate for increased intracellular thiol oxidation. Unchanged intracellular thiol levels (see results section 4.10) after diamide challenge support this statement. However, typical known "antioxidative" genes like *trxR*, *gpo* etc. were not differentially expressed in WT or Δ*tcyB*. Probably, the bacterium performs its reactions in different steps depending on the severity of the oxidative insult and related damages.

5.9 Predicted proteins involved in thiol- disulfide reactions in *L. sanfranciscensis*

The constraints chosen by Gopal et al. (2009) were also used in the present work to get a first overview of proteins involved in thiol- disulfide redox metabolism in *L. sanfranciscensis*. Additionally, proteins with CXXS and CXXT motif were chosen and analyzed.

Altogether 287 proteins with CXXC, CXXS or CXXT motif could be found in *L. sanfranciscensis.* Only four proteins out of 78 have a single CXXC motif near the N- terminus, a thioredoxin-fold and consist of less than 130 amino acids. The glutaredoxin-like protein *nrdH* has also one CXXT motif. Three out of these four proteins have already been annotated as glutaredoxin-like protein *nrdH*, thioredoxin-like protein *ytp* and thioredoxin. The other remaining hypothetical protein (LSA_02610) seemed to be a thiol- disulfide isomerase or thioredoxin with 50 – 68 % homology using BLASTP. Interestingly, the protein LSA_10270 with thioredoxin-fold, two CXXC and two CXXS motifs and with 189 amino acids in total, resulted in 30 – 40 % homology to a dithiol- disulfide isomerase in other gram- positive bacteria. It could be verified using 3D structure modeling that the typical thioredoxin-fold existed and that both cysteines of CXXC motif were located at the N- terminus of the α-helix. Thus, besides so far known proteins, LSA_02610 and LSA_10270 are possibly involved in thiol-disulfide redox metabolism in *L. sanfranciscensis* and require further investigations.

The other proteins with CXXC, a size smaller than 130 and no thioredoxin-fold include ribosomal proteins, LSA_02490 and LSA_03750. LSA_02490 seemed to be a cell wall anchor (cell surface) protein (30 – 40 % homology) whereas LSA_03750 is an uncharacterized hypothetical protein (63 – 81 % homology) as retrieved from BLASTP selecting only *Lactobacillales* as organisms.

The additional search for CXXS and CXXT motifs yielded no additional proteins as only two proteins with thioredoxin-fold (LSA_10270 for CXXS and LSA_04700 for CXXT) which were already selected using the CXXC motif could be obtained. This observation further reflects the importance of the proteins with CXXC motif in *L. sanfranciscensis*.

The content of Table 35 connects data received from transcriptome analysis with the results from the *in-silico* prediction protein analysis.

Table 35: Summarized table of predicted proteins which were significantly differentially expressed in transcriptome experiments using microarray and RNA- sequencing as investigated in the present work. Only proteins have been included which showed up- or downregulation (marked in grey with "yes") after microarray analysis and RNA- sequencing. The other proteins were omitted.

Gene id.	Description (annotation)	Microarray			RNA- seq
		Aerobic	Fructose	Candida	
LSA_04700	glutaredoxin-like protein nrdH	no	no	no	yes
LSA_08950	thioredoxin	no	no	yes	no
LSA_07350	peptide methionine sulfoxide reductase	no	no	yes	no

Gene id.	Description (annotation)	Microarray			RNA- seq
		Aerobic	Fructose	Candida	
LSA_04040	Bifunctional protein glmU	no	no	no	yes
LSA_13500	tRNA uridine 5- carboxylmethylaminomethyl modification enzyme mnmG	no	no	no	yes
LSA_11940	(3R)-hydroxymyristoyl-ACP dehydratase	yes	no	yes	no
LSA_06360	HTH- type transcriptional regulator YodB	no	no	yes	no
LSA_11930	Pyruvate carboxylase subunit A	no	no	yes	no
LSA_06310	50S ribosomal protein L32	no	yes	no	no
LSA_05840	30S ribosomal protein S14	no	yes	yes	no
LSA_02490	hypothetical protein	no	yes	no	no
LSA_11380	hypothetical protein	no	no	yes	no
LSA_10270	hypothetical protein	no	yes	yes	no
LSA_10290	hypothetical protein	no	no	no	yes
LSA_09320	hypothetical protein	yes	yes	no	no
LSA_2p00560	hypothetical protein	no	no	yes	no
LSA_06500	hypothetical protein	yes	no	yes	no
LSA_01110	hypothetical protein	yes	yes	yes	no

Most of the proteins with CXXC, CXXS and CXXT motif seemed to be differentially expressed when *L. sanfranciscensis* was co- cultured with *C. humilis* and in the presence of fructose. The hypothetical proteins LSA_02610 and LSA_03750 were not differentially expressed during the applied conditions which reflect that redox metabolism is complex with participation of small, not yet characterized proteins with possible diverse route of actions. The proteins LSA_02490 and LSA_10270 were downregulated when incubated with fructose (see A 18 in Appendix); the latter protein was upregulated after co- cultivation with *C. humilis* (see A 19 in Appendix). This evidences that also proteins larger than 130 amino acids can participate in thiol- disulfide reaction and that limitations of the used *in-silico* method have to be taken into account. Nevertheless, this approach illustrated an easy and cost-efficient way to screen available protein sequences without advanced knowledge in bioinformatics or the performance of extensive and time consuming experiments.

During the next step in characterization of "redox genes" in *L. sanfranciscensis*, possible gene targets for construction of knock-out mutants are given in this work. However, as redox reactions occur in many different ways and mostly intracellular, the establishment and application of suitable experimental methods which are sensitive and cost-saving should be a first objective.

6 SUMMARY

LAB are widely used as fermentation starters in the food industry. The application of suitable strains requires knowledge about their practical suitability during different environmental stress conditions. Different strain dependent mechanisms exist about the tolerance against diverse stressors like temperature, pH, pressure and presence of oxygen etc.

L. sanfranciscensis as typical wheat and rye sourdough bacterium comprises of a small genome. Despite this fact, the strain evolved a set of "antioxidative" genes which assist in the presence of variable stressors. So far the redox- active genes glutathione reductase (*gshR*) and NADH oxidase (*nox*) have already been characterized in this bacterium. They participate in thiol homeostasis and oxygen elimination.

The previous work should shed light on the importance and mode of actions of thioredoxin reductase (*trxR*), redox- sensing transcriptional repressor (*rex*), cystine transporter permease (*tcyB*) and glutathione peroxidase (*gpo*). For that purpose, knock-out construction caused by gene deletions was used to get insight into possible actions.

The distribution of genes involved in redox reactions in other LAB should provide knowledge about strains, which probably tolerate stress to a better extent. Also valuable information was required if there are any connections between the number of (positive) PCR signals and an (increased) tolerance against peroxide and thiol stress during growth.

Finally, transcriptional and *in-silico* protein prediction analysis were applied to identify unknown genes with possible participation in redox reactions.

The achieved mutants Δ*tcyB* and Δ*gpo* showed diminished growth during aerobiosis in media without manganese. Quantification revealed an increased production of H_2O_2 which probably accounted for an early entry into stationary phase. Thus, cystine transporter and glutathione peroxidase were both responsible for peroxide detoxification in *L. sanfranciscensis* amongst others.

Growth experiments of WT and Δ*tcyB* in mMRS and CDM were carried out to investigate the specificity of the cystine transporter. It can be stated that *tcyB* is the sole cystine transporter in *L. sanfranciscensis*. The transporter can also transport cysteine.

Shocking tests were conducted to receive information if the number of viable cells between WT and mutants is affected. Neither H_2O_2 nor diamide led to changes in the viable cell number in the exponential phase of growth. However, H_2O_2 treatment during this phase resulted equally in growth stagnation for WT and mutants. The extracellular thiol groups in Δ*tcyB* were increased after H_2O_2 treatment whereas intracellular thiol groups were lower compared to the WT. In contrast, extracellular thiol groups of the untreated Δ*gpo* mutant were increased. An elevated sensitivity against H_2O_2 and diamide could be seen using cells at the beginning of the exponential growth phase. Especially the

plate sensitivity assay revealed that Δ*tcyB* and Δ*gpo* were more sensitive to H_2O_2 than the WT and specifically Δ*tcyB* tolerates diamide to a lower extent in mMRS compared to the WT. The tolerance against reducing agents (cysteine, DTT and GSH) between WT and mutants was mainly comparable and reflects the strains ability to grow much better in the presence of reducing agents compared to growth with oxidizing agents.

Fermentations with measurement of ORP, pH and pO_2 showed no significant differences between WT, Δ*tcyB* and Δ*gpo*. Only Δ*tcyB* needed longer to reduce the present oxygen. The hypothesis of extracellular thiol groups which lead to a fall of ORP could be refused through application of the Δ*nox* mutant. Fermentations with Δ*nox* resulted in a positive ORP course although thiol group concentrations increased as high as for the WT, which showed a fall in ORP. Metabolite analysis was conducted to investigate possible differences in consumed substances or produced metabolites. The most remarkable differences were measured for ethanol and mannitol. An increased ethanol production after 24 hours of fermentation could be seen for Δ*gpo* and Δ*nox* due to increased NADH levels. Similarly, a significantly increased mannitol production could be measured for both strains although the initial fructose concentration was equal. A possible ROS scavenging mechanism in both mutants as described for other organisms could be one explanation besides an increase in NAD^+ generation.

The PCR screening of "redox" genes using degenerated primers should lead to first conclusions about the presence and distribution of chosen "redox" genes including "antioxidative" stress genes, peptidase, dehydrogenase, and genes involved in ETC in other LAB. It could be shown that the strains *L. plantarum*, *L. brevis*, *L. pontis* and *L. paracasei* had most positive results. Testing the growth of strains with a different number of positive PCR signals in the presence of oxidative agents revealed that only for two (*L. brevis*, *L. pontis*), out of the tested five groups, the results of PCR screening could be connected to the results from growth experiments. These give first insights into the complex route of "antioxidative" actions in LAB.

The transcriptional response of *L. sanfranciscensis* was investigated using microarray analysis after stimuli, which can occur during typical sourdough fermentation (presence of oxygen, fructose and *Candida humilis*). Predominantly, hypothetical proteins and pseudogenes were differentially expressed in all tested conditions which exemplified the potential role of so far uncharacterized proteins in a bacterium with a small genome. In the presence of oxygen, the oligopeptide transport system, *Clp* proteases and exinuclease protein *uvrABC* were upregulated wheras fatty acid synthesis was downregulated. The co- cultivation with *C. humilis* led to a similar transcriptional response (e.g. upregulation of *opp*) as in the presence of oxygen. Further, genes which are associated with stress (thioredoxin, *GroES*, *msrA*, *uvrABC*, *uspA*) were upregulated which assumes that *L. sanfranciscensis* was negatively influenced during co- cultivation with a maltose- negative yeast.

This work focused also on the transcriptional response after thiol stress caused by diamide in the WT and the generated Δ*tcyB*. The transcriptional response of the WT resembled the mechanisms which were known to occur after cold, heat and high pressure treatment because typical stress- associated genes were upregulated (*dnaJK*, *grpE*, *hrcA*, *groESL*, *Clp*, *ctsR*). The deletion in cystine transporter caused higher expression of *spx*, methionine transport and purine synthesis and again an upregulation of the *opp* operon. Probably, this transporter has other functions besides the replenishment of amino acids because that upregulation could already be detected after oxygen exposure and in the presence of *C. humilis*. A chaperone-like function or participation in import of signal peptides were proposed. Significantly differentially expressed isoforms could be identified.

Using *in-silico* protein prediction, proteins with CXXC, CXXS and CXXT motif near the N-terminus, a small size (< 130 amino acids) and a possible thioredoxin-fold from the published protein sequences of *L. sanfranciscensis* TMW 1.1304 were chosen. After analysis, two hypothetical proteins (LSA_02610, LSA_10270) out of 287 remained, with possible involvement in thiol- disulfide redox metabolism in *L. sanfranciscensis*.

The previous work evidenced that the cystine transporter TcyB in *L. sanfranciscensis* has an essential role during peroxide stress and in balancing the intracellular thiol homeostasis during thiol stress. Gpo participates in peroxide detoxification besides influencing the bacterial metabolism in direction to NAD^+ generation. Therefore, both proteins are involved in the redox metabolism in *L. sanfranciscensis*. The stress response of *L. sanfranciscensis* after different stimuli tested in this work overlapped with mechanisms described before. A tailor made stress response in this minimalistic bacterium could not be observed. Transcriptional analysis and *in-silico* protein prediction revealed that many hypothetical proteins with unknown function participated in redox reactions. Based on these results, the characterization of hypothetical proteins involved in redox reactions through knock-out construction in *L. sanfranciscensis* could be the next step in future applications.

7 ZUSAMMENFASSUNG

Milchsäurebakterien werden in großem Umfang als Starterkulturen in Fermentationen der Lebensmittelindustrie verwendet. Die Anwendung von geeigneten Stämmen setzt Wissen über deren praktische Anwendbarkeit bei unterschiedlichen Umweltbedingungen voraus. Es existieren verschiedene Stamm- abhängige Mechanismen, welche an der Toleranz gegen bestimmte Stressoren wie Temperatur, pH- Wert, Druck, Anwesenheit von Sauerstoff etc. beteiligt sind.

L. sanfranciscensis als typisches Bakterium, welches sehr häufig in Weizen und Roggensauerteigen zu finden ist, verfügt über ein sehr kleines Genom. Nichtsdestotrotz entwickelte dieser Stamm ein eigenes Set an „antioxidativen" Genen, welches ihn während des Einflusses von variablen Stressoren unterstützt. Bisher wurden die redoxaktiven Gene Glutathionreduktase (*gshR*) und NADH oxidase (*nox*) in diesem Bakterium charakterisiert. Diese sind am Thiolhaushalt und in der Sauerstoffelimierung beteiligt.

Die vorliegende Arbeit sollte die Bedeutung und Wirkungsweise der Gene Thioredoxinreduktase (*trxR*), Redox- sensing transkriptionaler Repressor (*rex*), Cystintransporter (*tcyB*) und Glutathione peroxidase (*gpo*) aufzeigen. Zu diesem Zweck sollten Deletionsmutanten konstruiert werden um Einblicke in vorliegende Mechanismen zu erhalten.

Weiterhin sollte die Verteilung von Redoxgenen in anderen Milchsäurebakterien Auskunft darüber geben, ob bestimmte Stämme mit einem höheren Set an „Redoxgenen" oxidativen Stress (Peroxid- und Thiolstress) besser tolerieren können oder nicht. Es sollte überprüft werden, ob die Ergebnisse des PCR- Screenings auf eine erhöhte Toleranz gegenüber Peroxid- und Thiolstress in Wachstumsversuchen übertragen werden könnnen.

Abschließend wurden Transkriptionsanalysen und *in-silico* Proteinsequenzanalysen angewendet um bisher unbekannte Gene zu identifizieren, welche an Redoxreaktionen beteiligt sein können.

Die Mutanten Δ*tcyB* und Δ*gpo* zeigten schlechteres Wachstumsverhalten in der Anwesenheit von Sauerstoff im Medium ohne Mangan. Eine erhöhte Bildung von H_2O_2 in den Mutanten konnte durch Quantifizierung von H_2O_2 gemessen werden, welches wahrscheinlich zu einem verfrühten Eintritt in die stationäre Phase führte. Demnach sind der Cystintransporter und die Glutathionperoxidase in *L. sanfranciscensis* u.a. für die Detoxifizierung von H_2O_2 verantwortlich.

Wachstumsexperimente des Wildtyps und der Δ*tcyB* Mutante in mMRS und CDM wurden durchgeführt, um die Spezifität des Cystintransporters zu bestimmen. Es konnte gezeigt werden, dass *tcyB* der einzigste Cystintransporter in *L. sanfranciscensis* ist. Dieser kann ebenfalls Cystein transportieren.

Durch Schockexperimente sollte untersucht werden, ob es Unterschiede zwischen WT und Mutanten in der Zahl an lebenden Zellen gibt. Weder H_2O_2 noch Diamid führten zu Veränderungen der

Lebendzellzahl in der exponentiellen Wachstumsphase. Die Behandlung mit H_2O_2 führte im WT, als auch den Mutanten, gleichermaßen zu Wachstumsstagnation. Die extrazellulären Thiolgruppen von Δ*tcyB* nach Behandlung mit H_2O_2 waren erhöht, wohingegen die intrazellulären Thiolgruppen, verglichen mit dem WT geringer waren. Demgegenüber waren die extrazellulären Thiolgruppen der unbehandelten Δ*gpo* Mutante erhöht. Eine zunehmende Sensitivität gegenüber H_2O_2 und Diamid konnte bei Zellen in der Anfangsphase des exponentiellen Wachstums beobachtet werden. Besonders der Plattensensitivitätsassay zeigte, dass Δ*tcyB* und Δ*gpo* sensitiver gegenüber H_2O_2 sind, verglichen mit dem WT. Speziell Δ*tcyB* toleriert Diamid im mMRS Medium weniger gut als der WT. Die Toleranz gegenüber reduzierenden Agenzien (Cystein, DTT und GSH) zwischen WT und Mutanten war im überwiegenden Maße vergleichbar und stellt die Fähigkeit der Stämme dar, sehr viel besser in der Anwesenheit von reduzierenden Agenzien zu wachsen, verglichen mit dem Wachstum in der Anwesenheit von oxidierenden Agenzien.

Fermentationen mit Messung des ORP, pH und pO_2 zeigten keine signifikanten Unterschiede zwischen WT, Δ*tcyB* und Δ*gpo*. Nur Δ*tcyB* brauchte länger um den verfügbaren Sauerstoff zu reduzieren. Die Hypothese, dass die Zunahme der extrazellulären Thiolgruppen zu einer Verringerung des ORP führt, konnte unter Verwendung der Δ*nox* Mutante nicht bestätigt werden. Die Fermentation mit Δ*nox* resultierte in einem positiven ORP Verlauf, obwohl die extrazellulären Thiolgruppen ähnlich anstiegen wie beim WT, welcher jedoch ein deutliches Absenken der Redoxpotenzialkurve zeigte. Um Unterschiede in konsumierten bzw. produzierten Substanzen zu erhalten, wurden ebenfalls die Metabolite analysiert. Die größten Unterschiede wurden für Ethanol und Mannitol gemessen. Eine Zunahme der Ethanolproduktion nach 24 Stunden konnte für Δ*gpo* and Δ*nox* beobachtet werden, welches wahrscheinlich das Ergebnis eines erhöhten NADH Pools ist. In ähnlicher Weise konnte eine signifikant erhöhte Mannitolproduktion für beide Stämme bestimmt werden, obwohl die initiale Fruktosekonzentration vergleichbar war. Neben einer erhöhten Generierung von NAD^+ könnte ein direkter Scavenging- Mechanismus der erzeugten ROS, wie bereits beschrieben für andere Organismen, eine weitere mögliche Erklärung sein.

Das PCR- Screening von „Redoxgenen“ unter Verwendung von degenerierten Primern sollte zu ersten Aussagen führen bezüglich der Ausstattung und Verbreitung von ausgewählten „Redoxgenen“, wie antioxidativen Stressgenen, Peptidasen, Dehydrogenasen und Genen der Elektronentransportkette in anderen Milchsäurebakterien. Stämme mit den meisten positiven PCR- Signalen waren *L. plantarum*, *L. brevis*, *L. pontis* und *L. paracasei*. Das Wachstum bestimmter Stämme mit einer unterschiedlichen Anzahl an positiven PCR- Signalen wurde in Anwesenheit von oxidativen Agenzien untersucht. Nur bei zwei (*L. brevis*, *L. pontis*) von den getesteten fünf Gruppen gab es Übereinstimmungen zwischen der Anzahl an positiven PCR- Signalen und dem Wachstum. Diese Ergebnisse erlauben erste Einblicke in die Komplexität „antioxidativer“ Mechanismen in Milchsäurebakterien.

Die Microarray- Analyse wurde angewendet, um die transkriptionale Antwort von *L. sanfranciscensis* auf bestimmte Stimuli zu erhalten, welche in typischen Sauerteigfermentationen vorkommen können (Anwesenheit von Sauerstoff, Fruktose und *C. humilis*). Die Mehrzahl der unterschiedlich exprimierten Gene kodierte für hypothetische Proteine sowie Pseudogene in allen getesteten Bedingungen, welches die potentielle Bedeutung der bisher uncharakterisierten Proteine in einem Bakterium mit einem sehr kleinen Genom veranschaulicht. In der Anwesenheit von Sauerstoff waren das Oligopeptidtransportsystem, die *Clp* Protease und die Exinuklease *uvrABC* hochreguliert, die Fettsäuresynthese hingegen runterreguliert. Die transkriptionale Antwort der Co- Kultivierung mit *C. humilis* ähnelte der in Anwesenheit von Sauerstoff (z. B. Hochregulation von *opp*). Weiterhin waren stress- assoziierte Gene (thioredoxin, *GroES*, *msrA*, *uvrABC*, *uspA*) hochreguliert, welche für einen negativen Einfluss der maltose – negativen Hefe auf *L. sanfranciscensis* sprechen.

Diese Arbeit beinhaltete ebenfalls die transkriptionale Antwort auf Thiolstress (hervorgerufen durch Diamid) des WT und der generierten Δ*tcyB* Mutante. Die transkriptionale Antwort des WT ähnelt den Mechanismen, die nach Kälte-, Hitze- und Hochdruckbehandlung induziert werden, da typische stress-assoziierte Gene hochreguliert waren (*dnaJK*, *grpE*, *hrcA*, *groESL*, *Clp*, *ctsR*). Die Deletion des Cystintransporters führte zu erhöhter Expression von *spx*, des Methionintransporters, der Purinsynthese und erneut von *opp*. Wahrscheinlich hat dieser Transporter neben der Anreicherung von Aminosäuren andere Funktionen, da eine Hochregulierung auch in der Anwesenheit von Sauerstoff und *C. humilis* detektiert werden konnte. Eine Chaperon- ähnliche Funktion sowie die Beteiligung am Import von Signalpeptiden erscheinen wahrscheinlich. Signifikant unterschiedlich exprimierte Isoformen wurden ebenfalls identifiziert.

Ausgehend von den veröffentlichten Proteinsequenzen von *L. sanfranciscensis* TMW 1.1304 wurden Proteine mit CXXC, CXXS und CSST Motiv am N- Terminus, mit einer Größe von < als 130 Aminosäuren und mit Thioredoxinfaltung ausgewählt. Zwei hypothetische Proteine (LSA_02610, LSA_10270) von insgesamt 287 verblieben, mit einer möglichen Beteiligung im Thiol- disulfid Redoxmetabolismus in *L. sanfranciscensis*.

Die vorliegende Arbeit stellt klar heraus, dass der Cystintransporter TcyB von *L. sanfranciscensis* eine essentielle Bedeutung bei der Detoxifizierung von Peroxiden und der Aufrechterhaltung der Thiolhomöostase hat. Gpo ist, neben der Beeinflussung des bakteriellen Metabolismusses in Richtung NAD^+ Bildung, an der Entgiftung von Peroxiden beteiligt. Beide Proteine sind demnach am Redoxmetabolismus von *L. sanfranciscensis* beteiligt. Die Stressantwort von *L. sanfranciscensis* nach verschiedenen Stimuli in dieser Arbeit überlappt mit bereits bekannten Reaktionen. Eine maßgeschneiderte Stressantwort in diesem minimalistischen Bakterium konnte nicht beobachtet werden. Sowohl die Transkriptionsanalysen, wie auch die Vorhersage an Thiol- Disulfidreaktionen beteiligter Proteine legen offen, dass viele hypothetische Proteine mit unbekannten Funktionen an Redoxreaktionen beteiligt sind. Ausgehend von diesen Ergebnissen kann in zukünftigen Experimenten

durch Knock-out Konstruktion eine weitere Charakterisierung ausgewählter hypothetischer Proteine, welche möglicherweise im Redoxmetabolismus von *L. sanfranciscensis* beteiligt sind, erfolgen.

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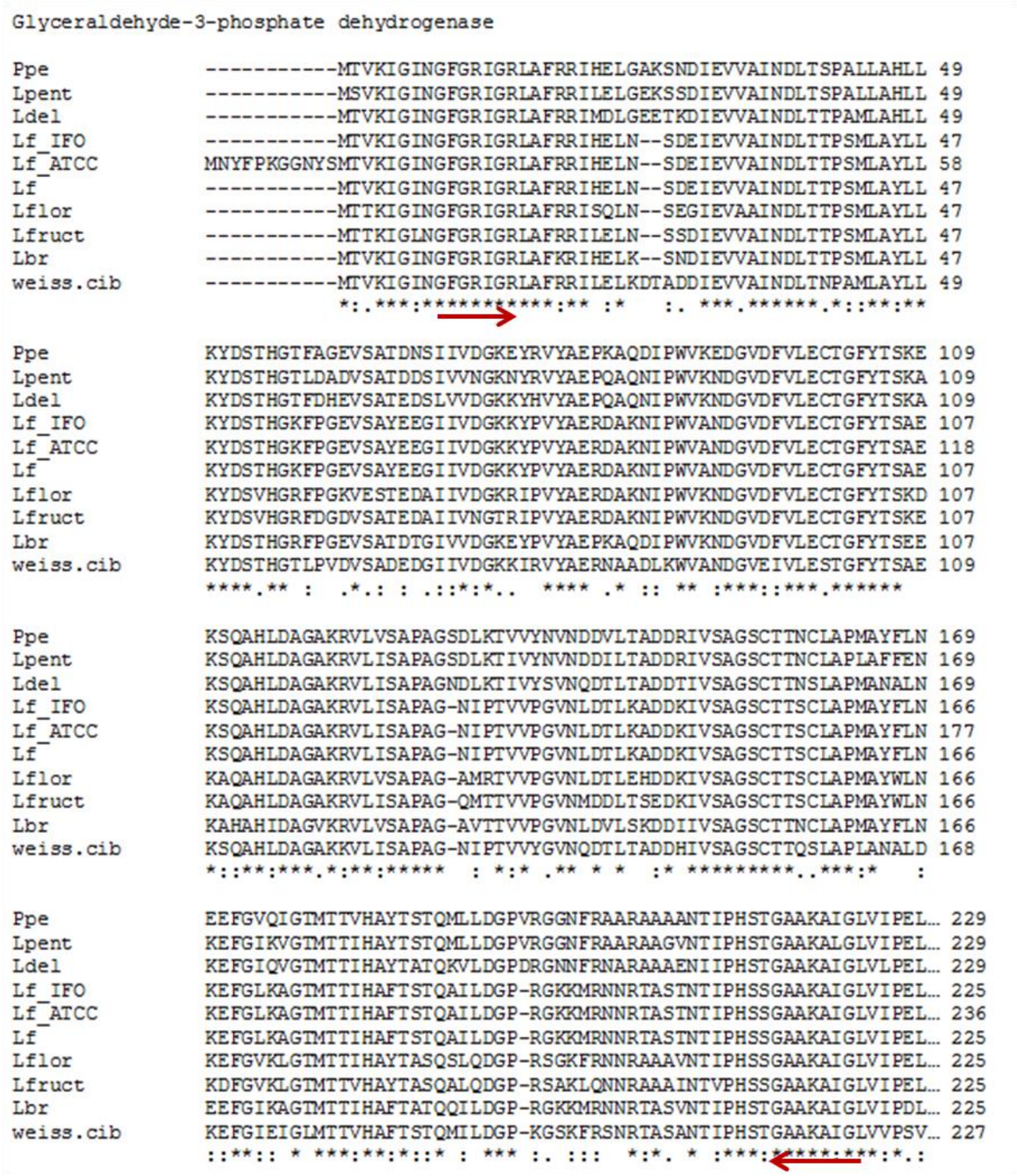
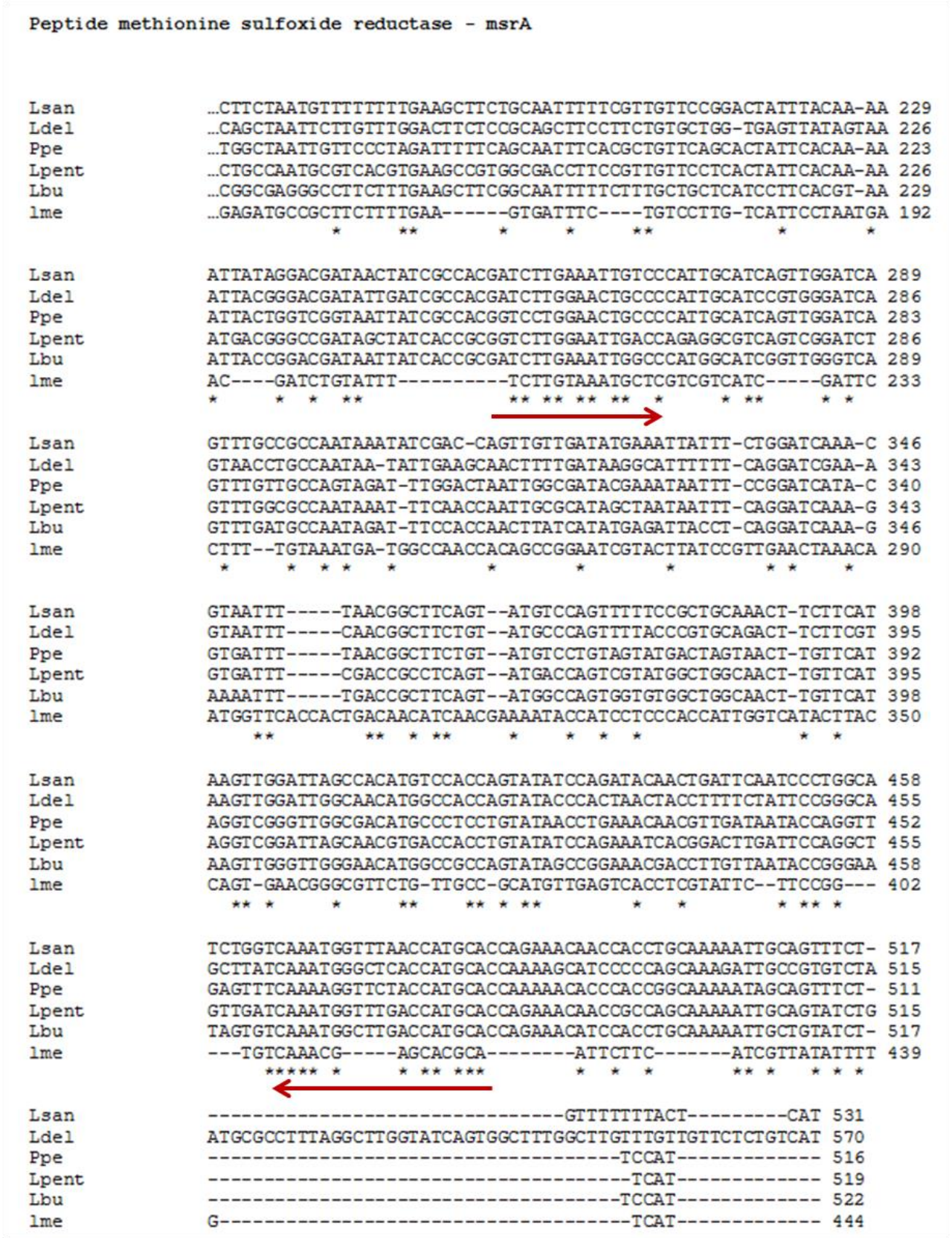
9 APPENDICES

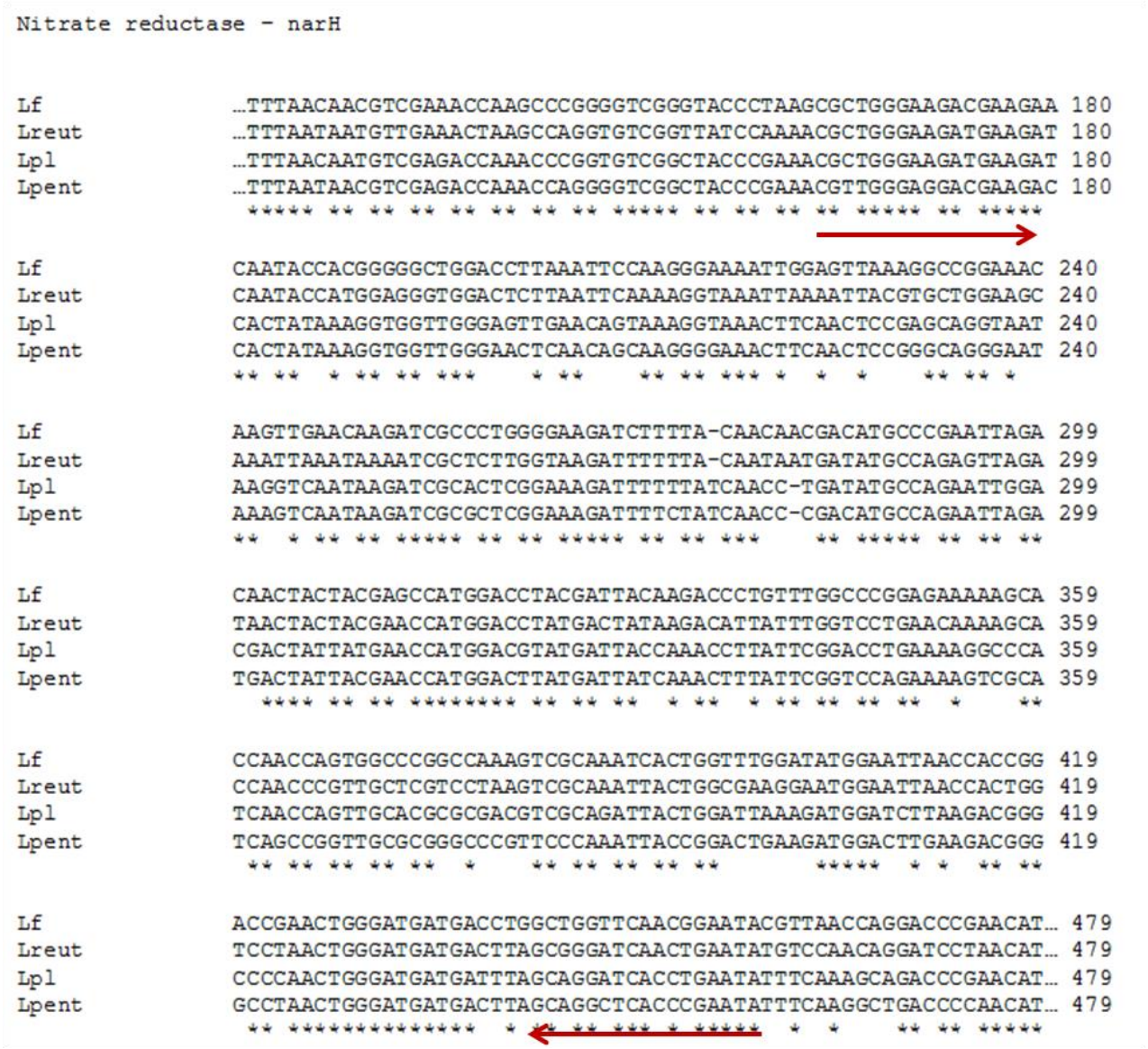
A 1: Multiple Alignment of DNA sequences of cytochrome B gene (*cytB*) using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Identical bases are marked with *, Because of the length of the sequences, the dashed line indicates the location of a gap; Red arrows mark the sites which were used for primer design.



A 2: Multiple Alignment of protein sequences of glyceraldehyde-3-phosphate dehydrogenase using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Identical bases are marked with *, Red arrows mark the sites which were used for primer design.

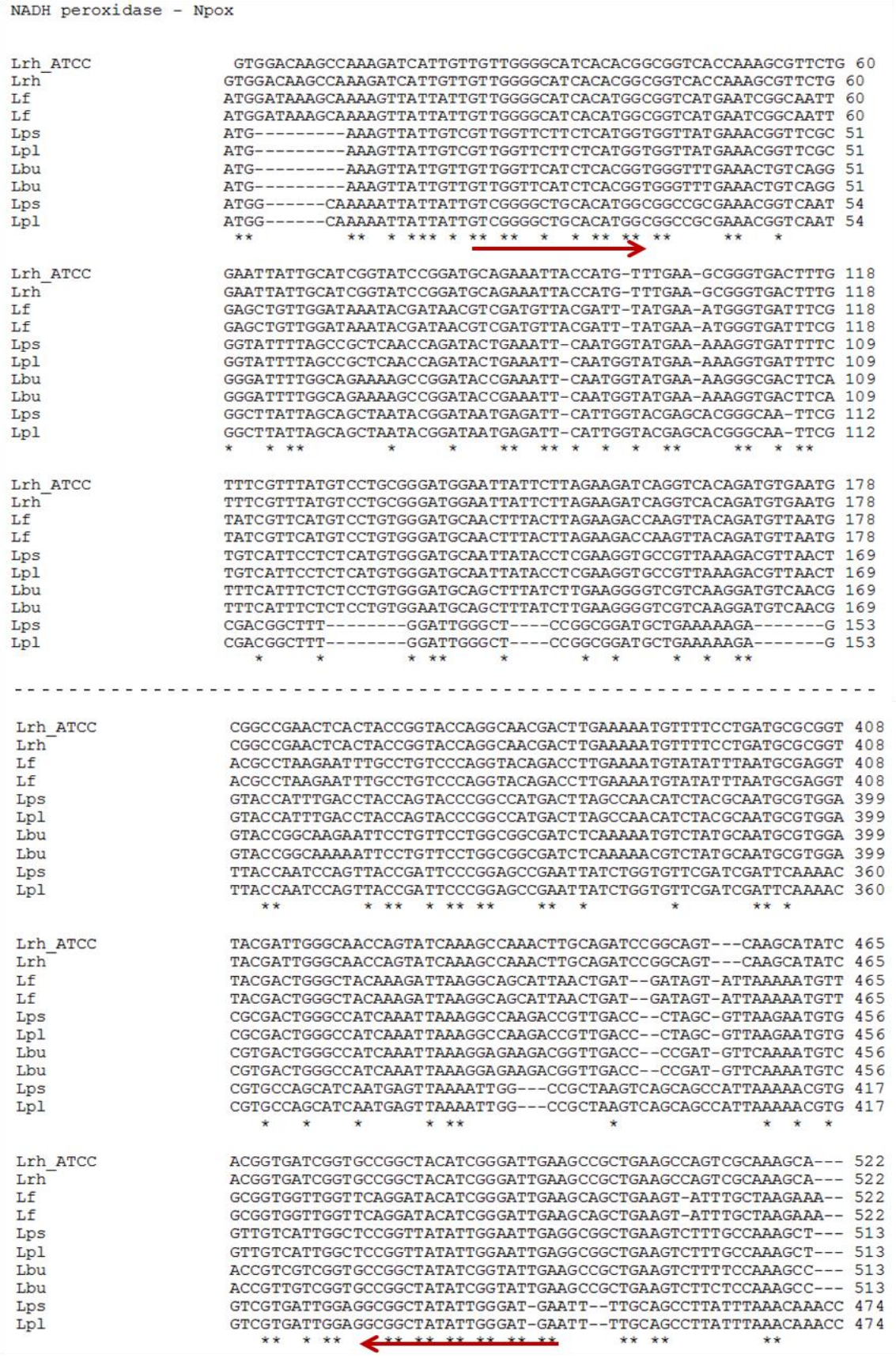


A 3: Multiple Alignment of DNA sequences of peptide methionine sulfoxide reductase gene (*msrA*) using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Identical bases are marked with *, Red arrows mark the sites which were used for primer design.

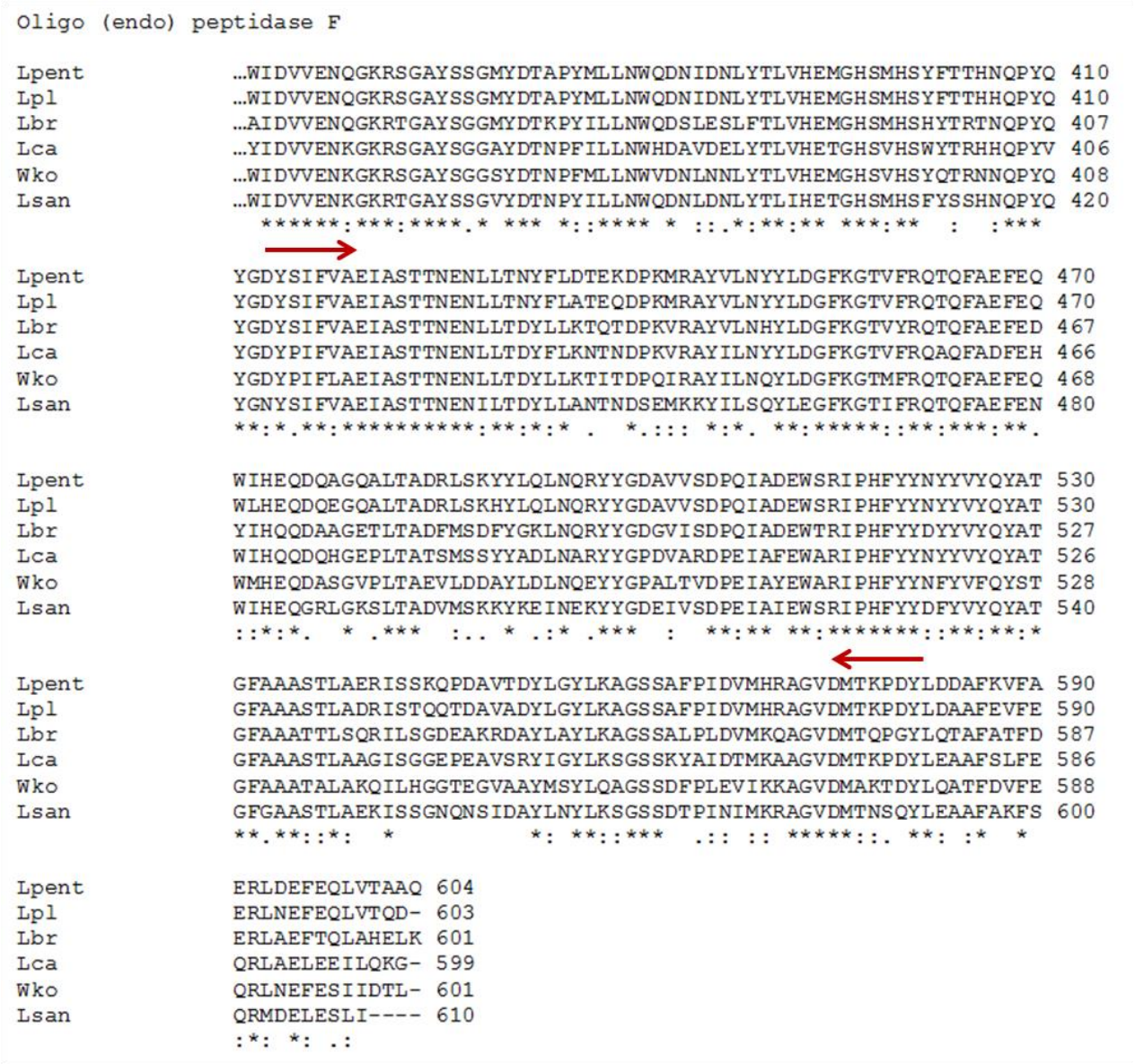


A 4: Multiple Alignment of DNA sequences of nitrate reductase (*narH*) using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Identical bases are marked with *, Red arrows mark the sites which were used for primer design.

A 5: Multiple Alignment of DNA sequences of NAD(P)H dehydrogenase (*Ndh2*) using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Identical bases are marked with *, Because of the length of the sequences, the dashed line indicates the location of a gap; Red arrows mark the sites which were used for primer design.

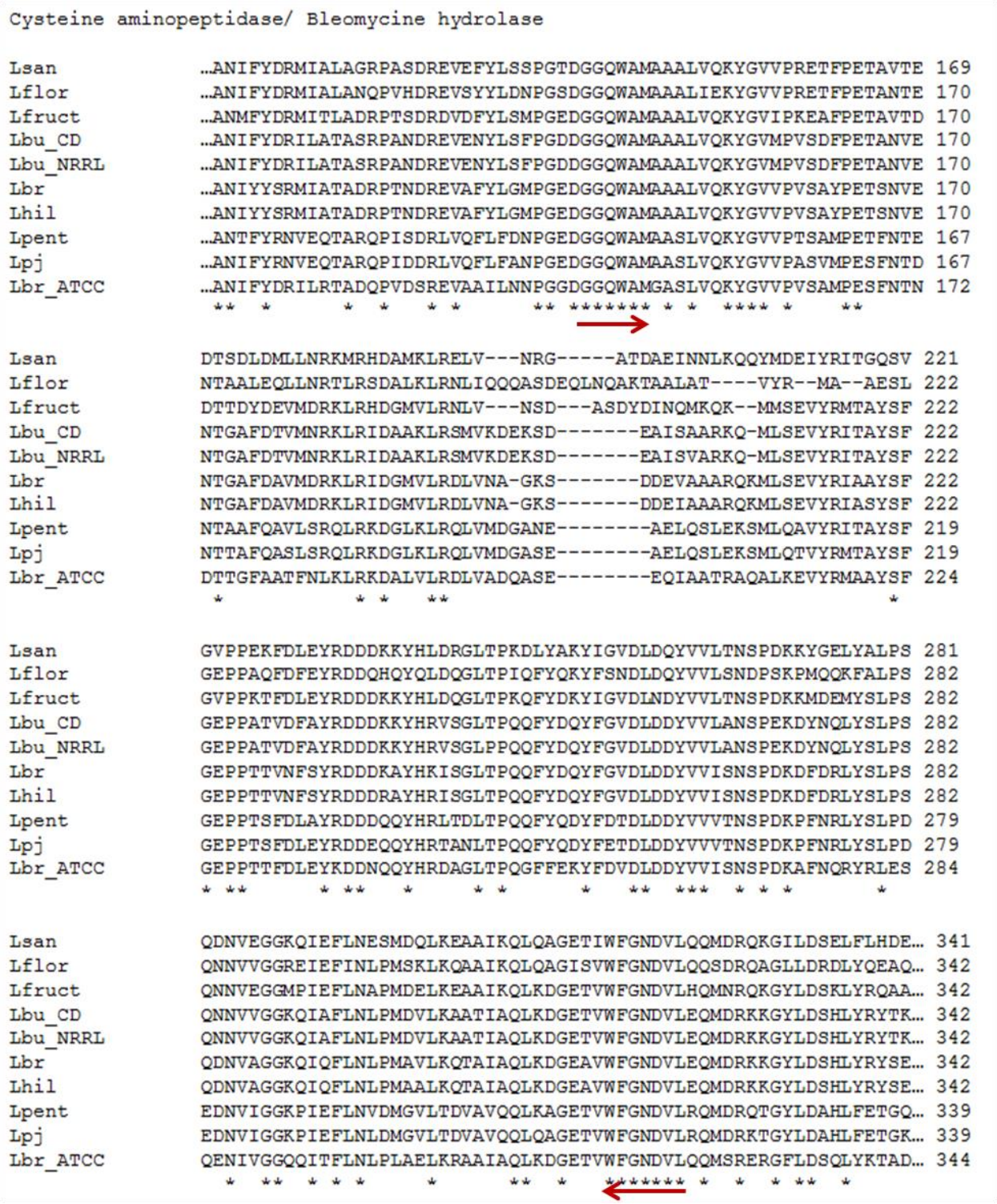


A 6: Multiple Alignment of DNA sequences of NADH peroxidase (*Npox*) using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Identical bases are marked with *, Because of the length of the sequences, the dashed line indicates the location of a gap; Red arrows mark the sites which were used for primer design.

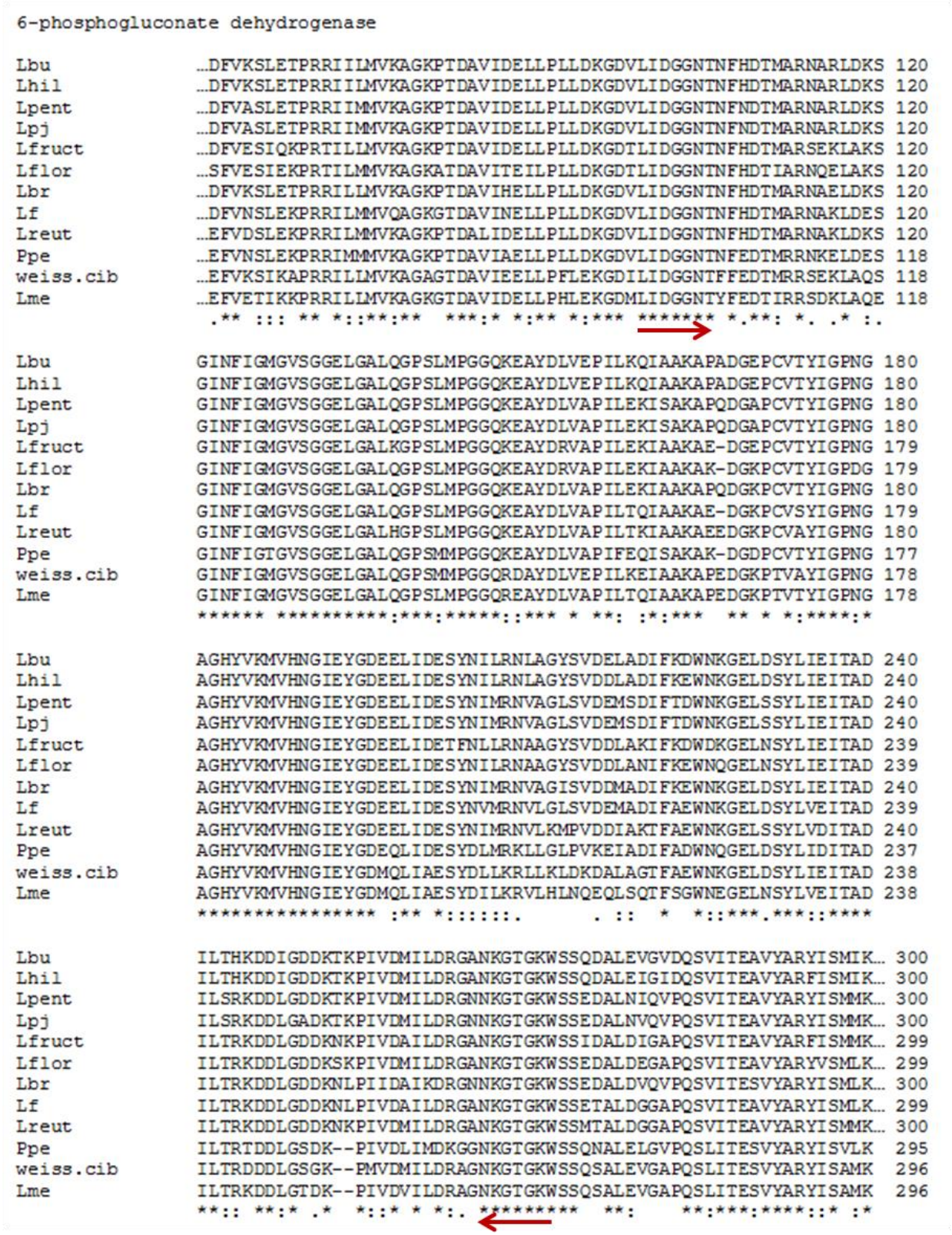


A 7: Multiple Alignment of protein sequences of oligo (endo) peptidase F using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Identical bases are marked with *, Red arrows mark the sites which were used for primer design.

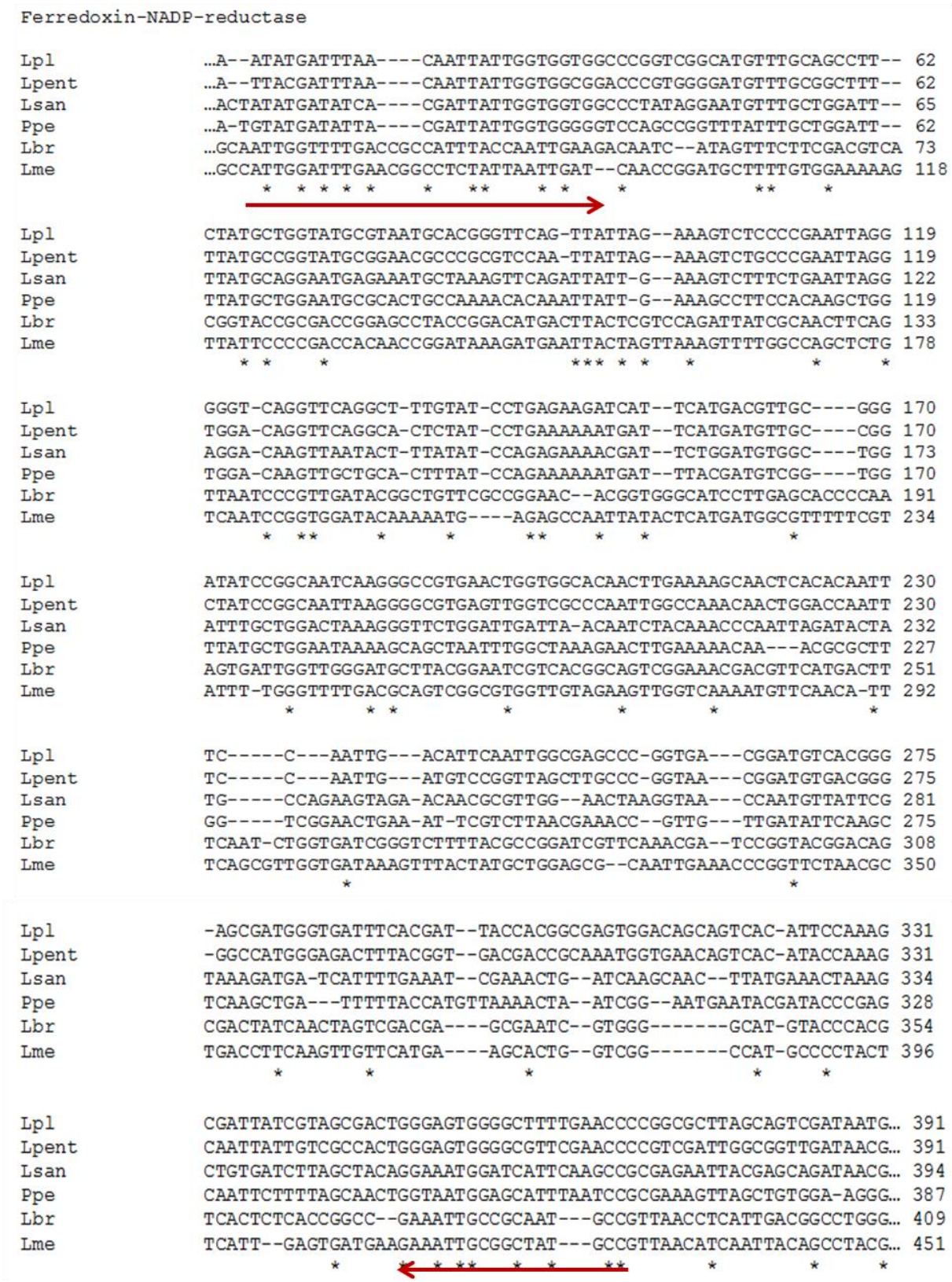
A 8: Multiple Alignment of protein sequences of aminopeptidase C using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Identical bases are marked with *, Red arrows mark the sites which were used for primer design.



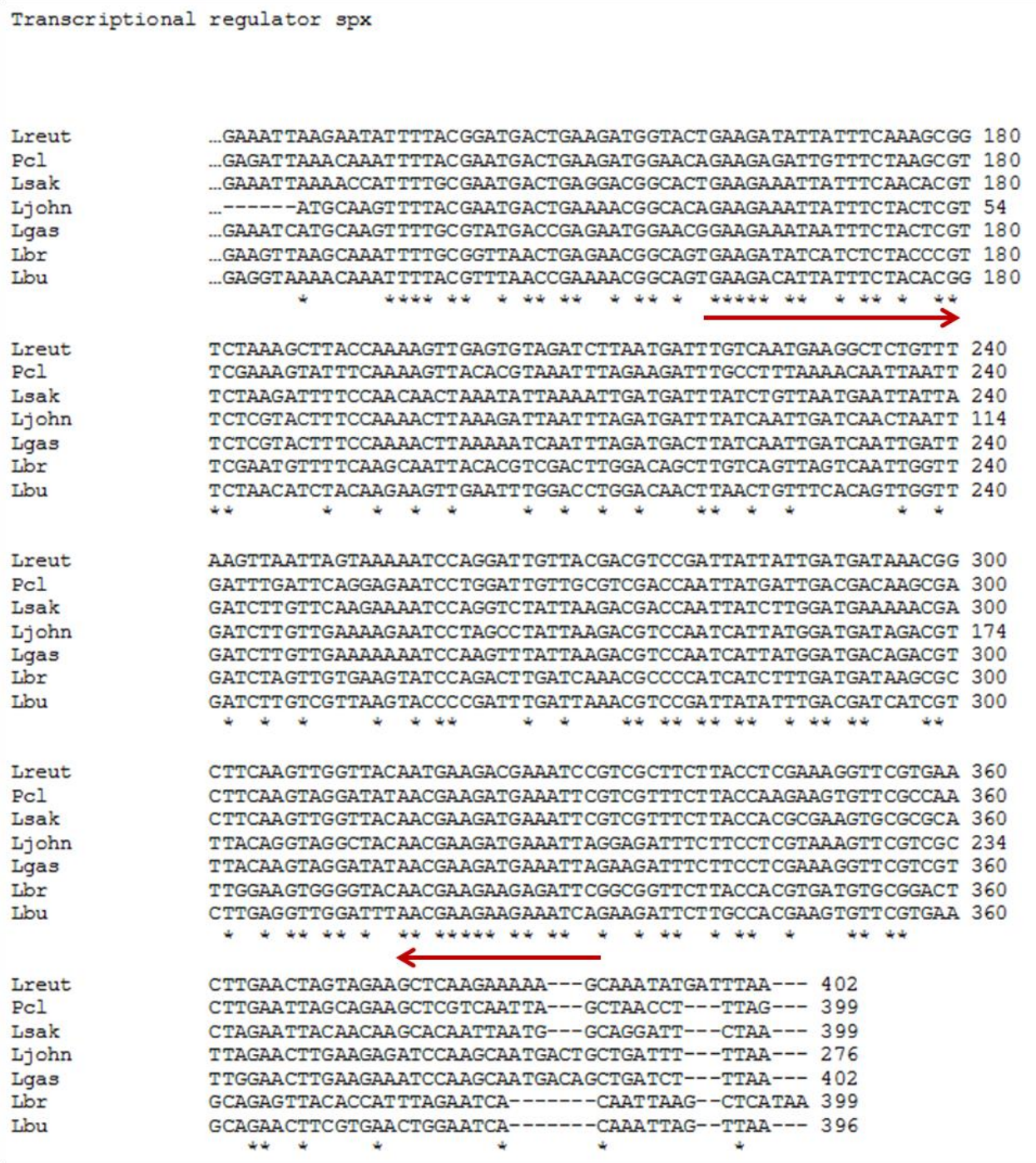
A 9: Multiple Alignment of protein sequences of cysteine aminopeptidase using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Identical bases are marked with *, Red arrows mark the sites which were used for primer design.



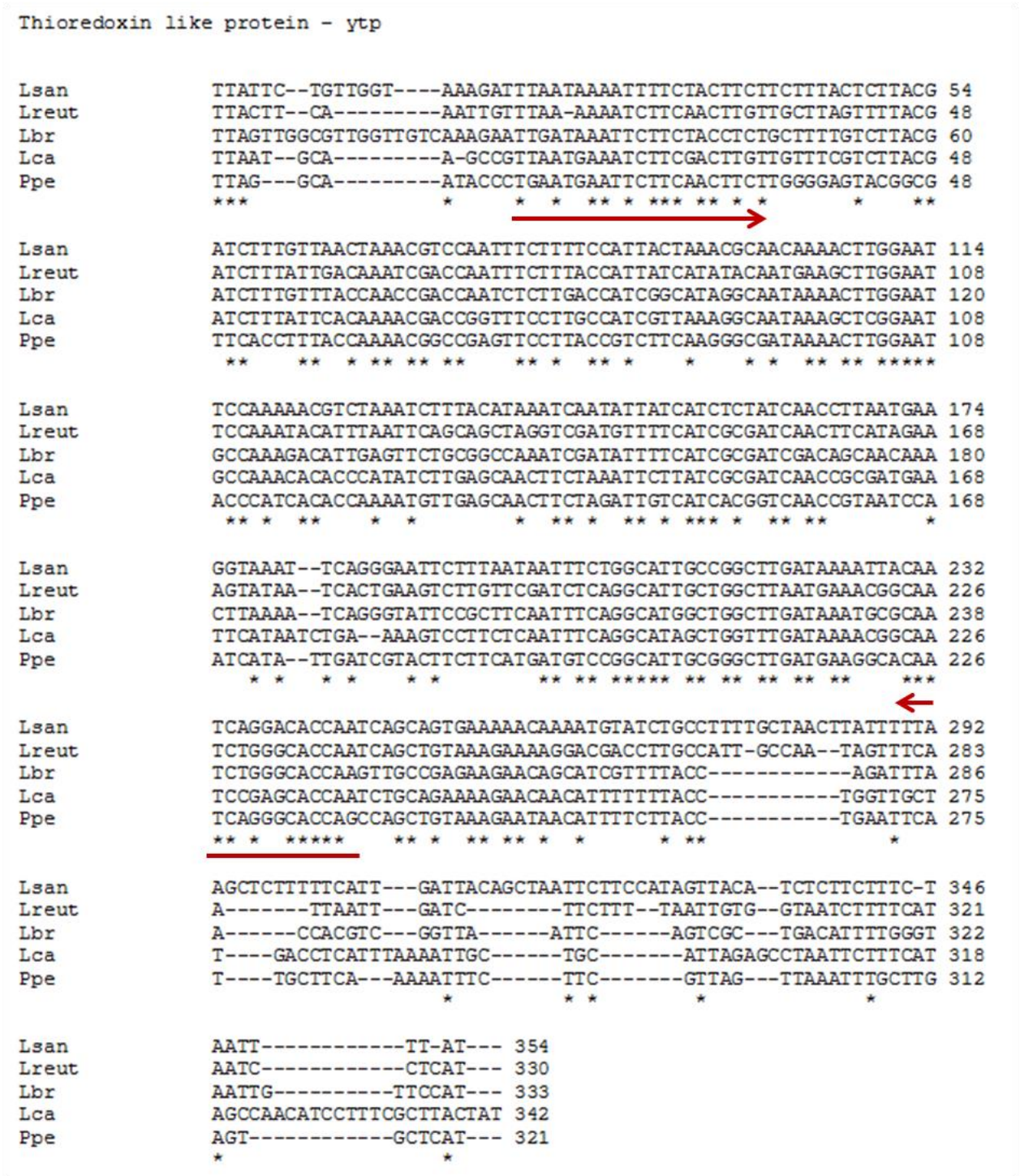
A 10: Multiple Alignment of protein sequences of 6-phosphogluconate dehydrogenase using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Identical bases are marked with *, Red arrows mark the sites which were used for primer design.



A 11: Multiple Alignment of DNA sequences of Ferredoxin-NADP-reductase using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Identical bases are marked with *, Red arrows mark the sites which were used for primer design.



A 12: Multiple Alignment of DNA sequences of transcriptional regulator *spx* using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Identical bases are marked with *, Red arrows mark the sites which were used for primer design.



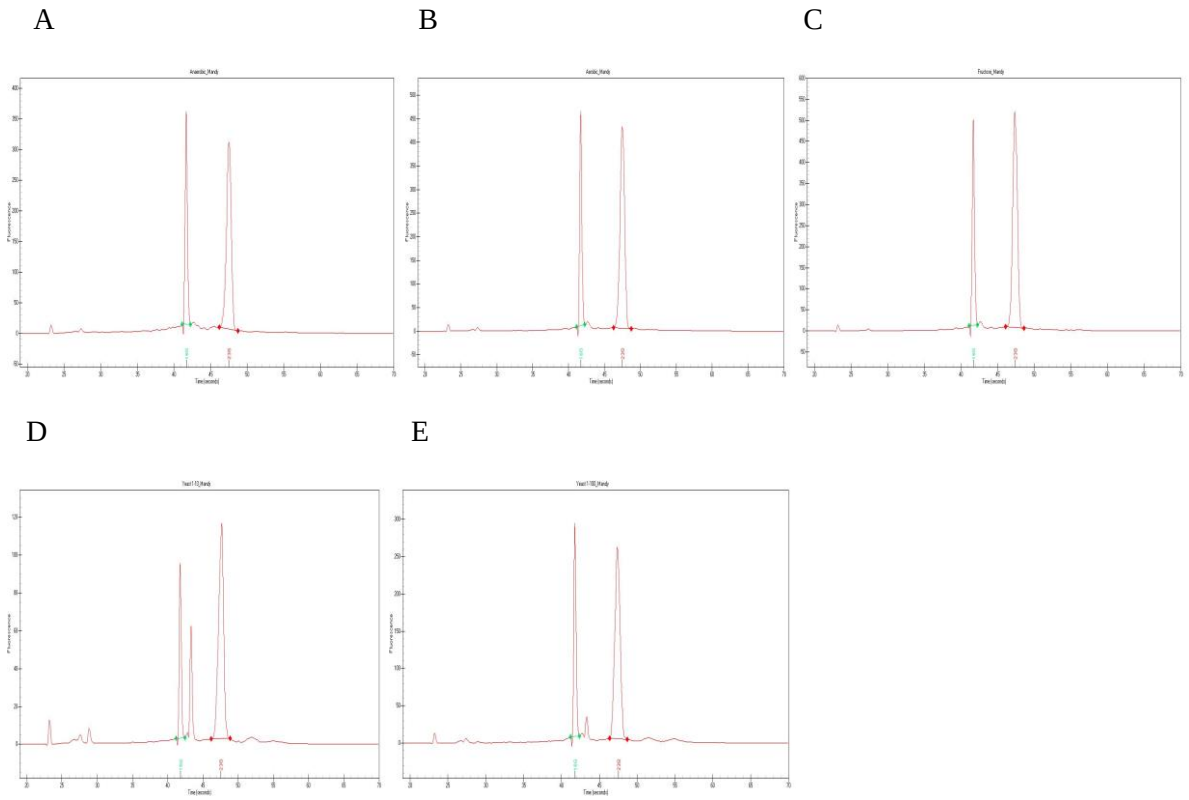
A 13: Multiple Alignment of DNA sequences of thioredoxin-like protein (*ytp*) using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Identical bases are marked with *, Red arrows mark the sites which were used for primer design.

tgatcTGatgGTncnCaATCACAGTTGTTTTTCCTTGTGGAAATTTAGCTGAAATTCCTTTGAGAACCGCTT
TTTtaCCAAAACTTTTTTTAATTTCATTAATTTTTAACATTAGTTCTTGTCCCCTTTCATATATCTAGA
AGACTTCTTTTCTAAGTAGCGTTGTAAGACGCTTAAAATTGTACATAGCATTGCATATAAAGCAGC
AACTTCAACATACATTAAAAGTGGTTCATAATTTTGTGCTGCATACTGCTGAATAATCTGAAACATT
TCAGCAATCGTAATGGTACTAGCTAATGATGTATCTTTAACCAAACCAATAAATTCGTTTGATAAA
GGTGGCAAAACAATTCGAAATGCTTGAGGCATTATAATTTTCCATAGTGTTTTGGGGGTACTAAAT
CCCAAAGTATATGCAGCATCCCACTGATCTTTATTAACTGATAACAAAGCTGAACGAATGGTTTCA
GAAGCATAAGCCCCAGTATTCAATGAAAAACCAATCACAGCTGCAGTGAAAGCTGGCAATTTAAC
CCCAATTGCTGGAAGACCAAAAAAGATAATAAATAATTGAACTAACAACGGTGTCGAACGAAAAA
CCCAGACATAAAAACTCGCAAGTAATCGTAGAAGTTTCCATAAAATTGAAACTATTCCACTAACCT
TTGGATTCAAAAATTTAATTAACGCTACAATTACAGCAATTATAATTCCAAATGTAAAAGAAAGTA
AGGTTAACGGGA**GGATCC**TCTAGAGTCGATTCACAAAAAaTAGGCACACGAAAAACAAGTTAAGG
GATGCAGTTTATGCATCCCTTAACTTACTTATTAAATAATTTATAGCTATTgaaAAGAGATAAGAATT
GTTCAAAGCtaATATTGTTTAAATCGTCAATTCCTGCATGTTTTAAGGAATTGTtaaATTGATTTTtTGT
AAATAttttcTTgtaTTCTTTGTTAACCCATTTCATAACGAAAtaATtataCTTttGTTTatcTTTgtgtgaacttTTTTTT
TCtaCtTAatcTGATaagtGAGCTATTCACTttAGgTTtagGatgAA

A 14: FASTA sequence of *L. sanfranciscensis* Δ*tcyB* after insertional inactivation. The primer pair ABC_for/ SP6 was used. Underlined is the disrupted *tcyB* gene, the restriction site (GGATCC) is marked in bold, the erythromycin resistance gene of *pME-1* is shaded in grey.

CTTTAGTTTCATTtATACccaATCATTTTTGTTTtaGGAATATATTTACCTAGTGAGATTTTATTAATTTT
ATTTATTTTTATTCTAATAACAGCATTCATCGGTGGTGTCATTTGGAAATTTTTAAATAATAATAATT
AATTTTTTCTTAATCAGTAATGTTCAATGCATCAATAATTTTTGGTTCAATCTTTAATGGAGAAGTT
ACTGGAGCATAGCGATGAATCAAGTTTCCCTTTTTTCCAATTAAAAATTTAGTATAATTCCATTTTA
TATTTCCGTGACCAGAAAGTCCTTTTAAATAAGTAAAAAGTGGAGATTCCTCATTTCCGTTTACTTT
TATCATTTCTGTCATTGGAAACGTAACCCCATAATGTATCTTACAATATTCATCGATATCTTTACTAT
CAAGTTCTTGATGAAATTGATTAGAAGGAAACCCAATTACTTCTAGTCCTTTTTGATGATATTTTTT
ATACAAATATTCAAGATTTTTTAATTGAGGAGCAAGCCCACATTTACTAGCCGTATTTACAACTAA
AATAACTTTATTTTTAAATTTAGTAAAATCAATTTCTCGACCATTCA**GGATCC**TCTAGAGTCGATTC
ACAAAAAATAGGCAACGAAAAACAAGTTAAGGGATGCAGTTTATGCATCCCTTAACTTACTTATTA
AATAATTTATAGCTATTGAAAAGAGATAAGAATTGTTCAAAGCTAATATTGTTTAAATCGTCAATT
CCTGCATGTTTTAAGGAATTGTTAAATTGATTTTTTGTAAATATTTTCTTGTATTCTTTGTTAACCCA
TTTCATAACGAAATAATTATACTTTTGTTTATCTTTGTGTGATATTCTTGATTTTTTTCTACTTAATCT
GATAAGTGAGCTATTCACTTTAGGTTTAGGATGAAAATATTCTCTTGGAACCATACTTAATATAGA
AATATCAACTTCTGCCATTAAAAGTAATGCCAATGAGCGTTtTGTATTTAAtAATCTTTTagcAAACCC
GTaTTCCACGATTaAATAAATCTCATTAGCTATACTATCAAAAaCAATTTtGCGTATTataTCCGTACTTa
tgttataagGTATATTACCAtataTTTtATAGGattggtTTTTAGgaAAtTTAAaCtgcaaTATATCCTTGTTTAAAACT
TGGAAATTAT

A 15: FASTA sequence of *L. sanfranciscensis* Δ*gpo* after insertional inactivation. The primer pair Pseu_for/ SP6 was used. Underlined is the disrupted *gpo* gene, the restriction site (GGATCC) is marked in bold, the erythromycin resistance gene of *pME-1* is shaded in grey.



A 16: Electropherograms of the isolated RNA samples after anaerobic (A) and aerobic (B) incubation, with added Fructose (C), after incubation 1/ 10 (D) and 1/ 100 (E) with *Candida humilis* for microarray analysis.

LSA number	COG functional category	Aerobic log2 fold changes	Up/ down	gene name, results of BLAST search
LSA_00600	Hypothetical proteins	1.6103	1	59 aa. hypothetical protein
LSA_00720	Hypothetical proteins	2.4832	1	mRNA interferase. cell growth regulatory protein (BLAST)
LSA_00740	Hypothetical proteins	0.4499	- 1	47 aa. hypothetical protein
LSA_00760	Hypothetical proteins	0.6239	- 1	102 aa. hypothetical protein
LSA_00790	Hypothetical proteins	0.579	- 1	56 aa. hypothetical protein BLAST
LSA_01040	Hypothetical proteins	1.8672	1	121 aa. hypothetical protein BLAST
LSA_01070	Hypothetical proteins	0.5826	- 1	142 aa. phosphatidylglycerophosphatase A BLAST
LSA_01110	**Hypothetical proteins**	**2.5635**	**1**	**transcriptional regulator (HTH. HrX. MarR) (BLAST)**
LSA_01300	Hypothetical proteins	3.4366	1	oxidoreductase (NADP dependent) iolS. inositol utilization protein (BLAST)
LSA_01320	**Hypothetical proteins**	**1.9537**	**1**	**136 aa. ribokinase. hypoth pr. transcr regulator BLAST**
LSA_01570	Hypothetical proteins	0.6456	- 1	195 aa. integrase family protein. recombinase plasmid associated
LSA_01940	Hypothetical proteins	0.541	- 1	170 aa. transcriptional regulator (TetR. ArsR) BLAST
LSA_01950	**Hypothetical proteins**	**0.6257**	**- 1**	**DegV family protein. hypothetical protein BLAST**
LSA_02310	Hypothetical proteins	0.5683	- 1	rRNA pseudouridine1911/1915/1917 synthase
LSA_02390	Hypothetical proteins	0.6402	- 1	135 aa. XRE family transcriptional regulator BLAST
LSA_02440	**Hypothetical proteins**	**3.342**	**1**	**42 aa. no information**
LSA_02510	Hypothetical proteins	1.5048	1	purine nucleosidase. inosine/uridine- preferring nucleoside hydrolase BLAST
LSA_02780	**Hypothetical proteins**	**6.4783**	**1**	**putative alcohol DH. zinc containing. oxidoreductase (BLAST)**
LSA_02800	Hypothetical proteins	0.5025	- 1	47 aa. hypothetical protein BLAST
LSA_02880	**Hypothetical proteins**	**0.4554**	**- 1**	**DegV family protein. hypothetical protein BLAST**
LSA_02900	Hypothetical proteins	1.924	1	150 aa. major facilitator superfamily protein BLAST
LSA_03010	Hypothetical proteins	0.6537	- 1	hypothetical protein
LSA_03040	Hypothetical proteins	2.8649	1	123 aa. hypothetical protein (XRE family DNA binding protein) BLAST
LSA_03320	**Hypothetical proteins**	**0.4738**	**- 1**	**superfamily II DNA/RNA helicase. DEAD/DEAH box helicase BLAST**
LSA_03370	Hypothetical proteins	0.6412	- 1	membrane protein. Cyclic nucleotide- binding domain-containing protein BLAST
LSA_03450	Hypothetical proteins	0.6571	- 1	153 aa.integral membrane protein BLAST
LSA_03460	**Hypothetical proteins**	**3.1607**	**1**	**179 aa. RNA polymerase (sigma SU) BLAST**
LSA_03510	**Hypothetical proteins**	**5.4251**	**1**	**67 aa. hypothetical protein. peptidyl- prolyl cis- trans isomerase BLAST**
LSA_04280	Hypothetical proteins	0.6516	- 1	147 aa. S1 RNA binding domain protein BLAST
LSA_04640	**Hypothetical proteins**	**0.3349**	**- 1**	**65 aa. hypothetical protein BLAST**
LSA_05600	**Hypothetical proteins**	**0.5801**	**- 1**	**115 aa. membrane protein. hypothetical protein BLAST**
LSA_05670	Hypothetical proteins	0.6453	- 1	teichoic acid/polysaccharide glycosyl transferase
LSA_05750	Hypothetical proteins	2.949	1	87 aa. hypothetical protein

LSA number	COG functional category	Aerobic log2 fold changes	Up/ down	gene name, results of BLAST search
LSA_05830	**Hypothetical proteins**	**0.5804**	**- 1**	**40 aa. hypothetical protein BLAST**
LSA_06500	Hypothetical proteins	1.9388	1	2- deoxyuridine 5- triphosphate nucleotidohydrolase. dUTPase BLAST
LSA_07300	**Hypothetical proteins**	**0.5888**	**- 1**	**155 aa. hypothetical protein BLAST**
LSA_07310	**Hypothetical proteins**	**3.3303**	**1**	**102 aa. hypothetical protein**
LSA_08100	**Hypothetical proteins**	**0.3777**	**- 1**	**hypothetical protein BLAST**
LSA_08500	**Hypothetical proteins**	**0.6202**	**- 1**	**rRNA methyltransferase (SAM dependent) BLAST**
LSA_08610	Hypothetical proteins	2.0209	1	186 aa. hypothetical protein BLAST
LSA_08660	**Hypothetical proteins**	**3.207**	**1**	**CRISPR assoc protein BLAST**
LSA_08810	**Hypothetical proteins**	**3.8391**	**1**	**144 aa. competence protein ComGF BLAST**
LSA_08820	**Hypothetical proteins**	**0.4868**	**- 1**	**56 aa. hypothetical protein BLAST**
LSA_08850	Hypothetical proteins	0.4394	- 1	competence protein ComGB BLAST
LSA_08860	**Hypothetical proteins**	**0.2713**	**- 1**	**competence protein ComGA BLAST**
LSA_08910	Hypothetical proteins	0.6356	- 1	small conductance mechanosensitive channel MscS
LSA_09320	Hypothetical proteins	0.546	- 1	XRE family transcriptional regulator (HTH domain containing protein)
LSA_10200	**Hypothetical proteins**	**0.3488**	**- 1**	**45 aa. hypothetical proteins BLAST**
LSA_10400	**Hypothetical proteins**	**0.6238**	**- 1**	**64 aa. hypothetical protein BLAST**
LSA_10560	Hypothetical proteins	0.629	- 1	membrane protein. ribonuclease BN-like family protein BLAST
LSA_10580	Hypothetical proteins	1.8716	1	144 aa. cell wall teichoic acid glycosylation protein BLAST
LSA_10650	**Hypothetical proteins**	**1.7836**	**1**	**97 aa. hypothetical protein BLAST**
LSA_11880	Hypothetical proteins	0.4876	- 1	179 aa. transcriptional regulator (TetR) BLAST
LSA_12030	Hypothetical proteins	0.5892	- 1	hypothetical protein BLAST
LSA_12080	Hypothetical proteins	0.6471	- 1	alpha/beta hydrolase. putative lipase BLAST
LSA_12230	Hypothetical proteins	2.2037	1	72 aa. hypothetical protein BLAST
LSA_12670	Hypothetical proteins	0.6231	- 1	transcriptional regulator. cell envelope- related transcriptional attenuator BLAST
LSA_13250	Hypothetical proteins	0.6395	- 1	transcriptional regulator LysR BLAST
LSA_1p00010	**Hypothetical proteins**	**0.6437**	**- 1**	**replication- associated protein RepB. plasmid copy control protein BLAST**
LSA_2p00160	**Hypothetical proteins**	**0.6584**	**- 1**	**41 aa. hypothetical protein BLAST**
LSA_2p00630	**Hypothetical proteins**	**0.8795**	**- 1**	**Cobyrinic acid ac- diamide synthase. replication-associated protein RepB BLAST**
LSA_00220	Pseudogenes	0.6421	- 1	pseudo
LSA_00230	Pseudogenes	0.6099	- 1	pseudo
LSA_00620	Pseudogenes	0.6493	- 1	pseudo
LSA_01310	Pseudogenes	3.0439	1	aldo/ ketoreductase family enzyme (NCBI BLASTN)
LSA_01470	Pseudogenes	0.6544	- 1	pseudo
LSA_01490	Pseudogenes	0.6006	- 1	pseudo
LSA_01930	Pseudogenes	0.649	- 1	pseudo
LSA_02050	Pseudogenes	1.6464	1	no information NCBI, KEGG

LSA number	COG functional category	Aerobic log2 fold changes	Up/ down	gene name, results of BLAST search
LSA_02120	**Pseudogenes**	**0.5622**	**- 1**	**pseudo**
LSA_02570	**Pseudogenes**	**2.7144**	**1**	**no information NCBI, KEGG**
LSA_03290	**Pseudogenes**	**3.4602**	**1**	**putative metal ion transporter (Mn) 16.5 kDa protein NCBI**
LSA_03670	**Pseudogenes**	**0.6247**	**- 1**	**pseudo**
LSA_04780	Pseudogenes	3.6782	1	putative ribose uptake protein. transcr regulator (NCBI)
LSA_04960	**Pseudogenes**	**0.2796**	**- 1**	**pseudo**
LSA_04970	**Pseudogenes**	**0.3318**	**- 1**	**pseudo**
LSA_05050	**Pseudogenes**	**2.6247**	**1**	**DNA / RNA helicase for DNA uptake (NCBI)**
LSA_05690	**Pseudogenes**	**0.2211**	**- 1**	**pseudo**
LSA_05700	Pseudogenes	0.6442	- 1	pseudo
LSA_05740	Pseudogenes	1.6585	1	pseudo
LSA_06920	**Pseudogenes**	**1.6487**	**1**	**pseudo**
LSA_08060	**Pseudogenes**	**0.6547**	**- 1**	**pseudo**
LSA_08770	**Pseudogenes**	**0.1776**	**- 1**	**acetate kinase uncharact transporter. transposase NCBI**
LSA_08780	**Pseudogenes**	**0.5753**	**- 1**	**pseudo**
LSA_11570	Pseudogenes	1.7861	1	pseudo
LSA_11710	Pseudogenes	0.4351	- 1	pseudo
LSA_12370	**Pseudogenes**	**2.5668**	**1**	**no information NCBI, KEGG**
LSA_12520	**Pseudogenes**	**0.6465**	**- 1**	**62 bp of lactate dehydrogenase NCBI**
LSA_13120	Pseudogenes	0.5813	- 1	pseudo
LSA_13210	**Pseudogenes**	**1.92**	**1**	**pseudo**
LSA_13220	**Pseudogenes**	**5.0956**	**1**	**no information NCBI, KEGG**
LSA_1p00110	Pseudogenes	0.6289	- 1	pseudo

A 17: Differentially expressed genes expressed as log2 fold changes during aerobic incubation of *L. sanfranciscensis* TMW 1.1304 ordered alphabetically by COG functional categories. Anaerobic conditions were used as reference condition and set as 1. Up- regulated genes are shaded in grey, downregulated genes are left blank. Genes which were also differentially expressed during incubation with fructose are marked bold.

LSA number	COG functional category	Fructose log2 fold changes	Up/down	gene name. results of BLAST search
LSA_00510	Hypothetical proteins	3.761	1	glucosyltransferase (Strepmut). serine endopeptidase BLAST
LSA_00740	Hypothetical proteins	0.348	- 1	47 aa. hypothetical protein BLAST
LSA_00790	Hypothetical proteins	1.5299	1	56 aa. hypothetical protein BLAST
LSA_01030	Hypothetical proteins	1.5932	1	168 aa. abortive infection protein. immunity protein BLAST
LSA_01100	Hypothetical proteins	2.8236	1	HxlR transcriptional regulator BLAST
LSA_01110	**Hypothetical proteins**	**2.8763**	**1**	**transcriptional regulator (HTH. HrX. MarR) (BLAST)**
LSA_01140	Hypothetical proteins	0.6473	- 1	142 aa. methylenomycin A resistance protein BLAST
LSA_01290	Hypothetical proteins	0.64	- 1	172 aa. hypothetical protein BLAST
LSA_01320	**Hypothetical proteins**	**1.8106**	**1**	**136 aa. transcr regulator (MarR). hypothetical protein**
LSA_01560	Hypothetical proteins	0.5865	- 1	120 aa. hypothetical protein BLAST
LSA_01950	**Hypothetical proteins**	**0.6149**	**- 1**	**DegV family protein. hypothetical protein BLAST**
LSA_02440	**Hypothetical proteins**	**2.8611**	**1**	**42 aa. no information**
LSA_02480	Hypothetical proteins	1.5459	1	75 aa. hypothetical protein BLAST
LSA_02490	Hypothetical proteins	0.5168	- 1	61 aa. LPXTG- motif cell wall anchor domain- containing protein

LSA number	COG functional category	Fructose log2 fold changes	Up/down	gene name. results of BLAST search
LSA_02780	**Hypothetical proteins**	**2.4346**	**1**	**putative alcohol DH. zinc containing. oxidoreductase (BLAST)**
LSA_02880	**Hypothetical proteins**	**0.5591**	**- 1**	**DegV family protein. hypothetical protein BLAST**
LSA_02890	Hypothetical proteins	1.6814	1	50 aa. hypothetical protein BLAST
LSA_02910	Hypothetical proteins	0.6495	- 1	44 aa. hypothetical protein BLAST
LSA_02990	Hypothetical proteins	1.7451	1	80 aa. hypothetical protein BLAST
LSA_03200	Hypothetical proteins	0.4615	- 1	48 aa. hypothetical protein BLAST
LSA_03320	**Hypothetical proteins**	**0.5406**	**- 1**	**superfamily II DNA/RNA helicase. DEAD/DEAH box helicase BLAST**
LSA_03460	**Hypothetical proteins**	**3.2075**	**1**	**179 aa. RNA polymerase (sigma SU) BLAST**
LSA_03470	Hypothetical proteins	5.435	1	peptidyl- prolyl cis- trans isomerase (Proteinfaltung) BLAST
LSA_03480	Hypothetical proteins	2.0047	1	hypothetical protein BLAST
LSA_03510	**Hypothetical proteins**	**3.234**	**1**	**67 aa. hypothetical protein. peptidyl- prolyl cis- trans isomerase BLAST**
LSA_04640	**Hypothetical proteins**	**1.6512**	**1**	**65 aa. hypothetical protein**
LSA_05380	Hypothetical proteins	0.541	- 1	157 aa. ATP- binding protein. ATP/GTP hydrolase. Kinase BLAST
LSA_05390	Hypothetical proteins	0.5025	- 1	160 aa. acetyltransferase (GNAT family) BLAST
LSA_05500	Hypothetical proteins	0.6454	- 1	104 aa. hypothetical protein BLAST
LSA_05600	**Hypothetical proteins**	**0.4554**	**- 1**	**115 aa. membrane protein. hypothetical protein BLAST**
LSA_05830	**Hypothetical proteins**	**0.5062**	**- 1**	**40 aa. hypothetical protein BLAST**
LSA_05960	Hypothetical proteins	0.5753	- 1	110 aa. hypothetical protein BLAST
LSA_05990	Hypothetical proteins	0.6174	- 1	115 aa. hypothetical protein BLAST
LSA_06070	Hypothetical proteins	0.6218	- 1	104 aa. hypothetical protein BLAST
LSA_06470	Hypothetical proteins	0.5705	- 1	137 aa. rhodanese- related sulfurtransferase. rhodanese family protein BLAST
LSA_07140	Hypothetical proteins	0.5775	- 1	195 aa. hypothetical protein BLAST
LSA_07270	Hypothetical proteins	0.5597	- 1	DegV family protein BLAST
LSA_07300	**Hypothetical proteins**	**0.4167**	**- 1**	**155 aa. hypothetical protein BLAST**
LSA_07310	**Hypothetical proteins**	**1.6127**	**1**	**102 aa. hypothetical protein**
LSA_07690	Hypothetical proteins	0.6588	- 1	62 aa. hypothetical protein BLAST
LSA_07880	Hypothetical proteins	0.5868	- 1	N- acetylmuramoyl- L- alanine amidase. cell wall hydrolase/autolysin BLAST
LSA_07940	Hypothetical proteins	0.6156	- 1	102 aa. hypothetical protein BLAST
LSA_08040	Hypothetical proteins	1.9097	1	42 aa. hypothetical protein BLAST
LSA_08100	**Hypothetical proteins**	**5.1912**	**1**	**hypothetical protein BLAST**
LSA_08470	Hypothetical proteins	0.65	- 1	Carbohydrate kinase. YjeF-like protein. hypothetical protein BLAST
LSA_08500	**Hypothetical proteins**	**0.6506**	**- 1**	**rRNA methyltransferase (SAM dependent) BLAST**
LSA_08660	**Hypothetical**	**1.701**	**1**	**CRISPR assoc protein BLAST**

LSA number	COG functional category	Fructose log2 fold changes	Up/down	gene name. results of BLAST search
	proteins			
LSA_08690	Hypothetical proteins	0.6501	- 1	179 aa. inner membrane protein. membrane- bound metal-dependent hydrolase BLAST
LSA_08810	**Hypothetical proteins**	**1.9262**	**1**	**144 aa. competence protein ComGF BLAST**
LSA_08820	**Hypothetical proteins**	**0.464**	**- 1**	**56 aa. hypothetical protein BLAST**
LSA_08830	Hypothetical proteins	4.9988	1	competence protein ComGD BLAST
LSA_08840	Hypothetical proteins	5.6659	1	competence protein ComGC BLAST
LSA_08860	**Hypothetical proteins**	**6.3271**	**1**	**competence protein ComGA BLAST**
LSA_08900	Hypothetical proteins	0.6035	- 1	127 aa. methyl- accepting chemotaxis-like protein. hypothetical protein BLAST
LSA_09300	Hypothetical proteins	0.5724	- 1	94 aa. integral membrane protein. hypothetical protein BLAST
LSA_09320	Hypothetical proteins	0.6155	- 1	XRE family transcriptional regulator. HTH protein BLAST
LSA_09890	Hypothetical proteins	0.5484	- 1	153 aa. hypothetical protein. membrane protein BLAST
LSA_10200	**Hypothetical proteins**	**0.3532**	**- 1**	**45 aa. hypothetical proteins BLAST**
LSA_10270	Hypothetical proteins	0.6489	- 1	189 aa. dithiol- disulfide isomerase. hypothetical protein BLAST
LSA_10320	Hypothetical proteins	0.586	- 1	166 aa. hypothetical protein. monooxygenase BLAST
LSA_10400	**Hypothetical proteins**	**0.5983**	**- 1**	**64 aa. hypothetical protein BLAST**
LSA_10720	Hypothetical proteins	1.6564	1	47 aa. protease synthase and sporulation negative regulating protein. GNAT family acetyltransferase BLAST
LSA_11400	Hypothetical proteins	0.5137	- 1	81 aa. membrane protein. hypothetical protein BLAST
LSA_11560	Hypothetical proteins	0.6078	- 1	47 aa. amidase. hypothetical protein BLAST
LSA_12460	Hypothetical proteins	1.5051	1	hypothetical protein BLAST
LSA_12570	Hypothetical proteins	0.6471	- 1	UDP- D- galactose:(glucosyl)LPS alpha- 1.6- D-galactosyltransferase BLAST
LSA_12690	Hypothetical proteins	0.4631	- 1	60 aa. hypothetical protein BLAST
LSA_1p00010	**Hypothetical proteins**	**0.6428**	**- 1**	**replication- associated protein RepB BLAST**
LSA_1p00020	Hypothetical proteins	0.6529	- 1	112 aa. hypothetical protein BLAST
LSA_1p00030	Hypothetical proteins	0.6498	- 1	hypothetical protein. transposase BLAST
LSA_2p00090	Hypothetical proteins	0.5827	- 1	40 aa. hypothetical protein BLAST
LSA_2p00150	Hypothetical proteins	0.5849	- 1	47 aa. hypothetical protein. short- chain dehydrogenase/oxidoreductase. 3- beta hydroxysteroid dehydrogenase
LSA_2p00160	**Hypothetical proteins**	**0.628**	**- 1**	**41 aa. hypothetical protein BLAST**
LSA_2p00360	Hypothetical proteins	2.3084	1	hypothetical protein BLAST
LSA_2p00630	**Hypothetical proteins**	**0.872**	**- 1**	**Cobyrinic acid ac- diamide synthase. plasmid copy control protein. replication- associated protein RepB**
LSA_02120	**Pseudogenes**	**0.5999**	**- 1**	**pseudo**
LSA_02570	**Pseudogenes**	**2.6589**	**1**	**no information NCBI. KEGG**
LSA_02670	Pseudogenes	2.2549	1	mannitol/ chloraromatic transport system. H+/ gluconate transporter NCBI
LSA_03290	**Pseudogenes**	**2.5215**	**1**	**putative metal ion transporter (Mn) 16.5 kDa protein NCBI**

LSA number	COG functional category	Fructose log2 fold changes	Up/down	gene name. results of BLAST search
LSA_03670	**Pseudogenes**	**0.6164**	**- 1**	**pseudo**
LSA_04960	**Pseudogenes**	**3.4054**	**1**	**10 kDa Chaperonin (NCBI). ABC transporter NCBI**
LSA_04970	**Pseudogenes**	**14.2324**	**1**	**10 kDa Chaperonin (NCBI). ABC transporter NCBI**
LSA_05040	Pseudogenes	2.458	1	competence protein. helicase domain protein
LSA_05050	**Pseudogenes**	**2.7144**	**1**	**DNA / RNA helicase for DNA uptake (NCBI)**
LSA_05210	Pseudogenes	1.5267	1	pseudo
LSA_05220	Pseudogenes	1.9509	1	pseudo
LSA_05470	Pseudogenes	0.5956	- 1	pseudo
LSA_05690	**Pseudogenes**	**0.5296**	**- 1**	**pseudo**
LSA_05860	Pseudogenes	0.6542	- 1	pseudo
LSA_06920	**Pseudogenes**	**1.6745**	**1**	**pseudo**
LSA_07340	Pseudogenes	2.0916	1	ABC transporter. permease protein. peptide methionine sulfoxide reductase BLAST
LSA_08000	Pseudogenes	2.0673	1	no information NCBI. KEGG
LSA_08770	**Pseudogenes**	**2.717**	**1**	**acetate kinase uncharact transporter, transposase NCBI**
LSA_08780	**Pseudogenes**	**1.5549**	**1**	**pseudo**
LSA_12370	**Pseudogenes**	**2.6436**	**1**	**no information NCBI, KEGG**
LSA_12520	**Pseudogenes**	**5.4422**	**1**	**62 bp of lactate dehydrogenase NCBI**
LSA_12730	Pseudogenes	1.8337	1	pseudo
LSA_13210	**Pseudogenes**	**2.3223**	**1**	**no information NCBI, KEGG**
LSA_13220	**Pseudogenes**	**2.7936**	**1**	**no information NCBI, KEGG**
LSA_13230	Pseudogenes	4.174	1	UPF0324 membrane protein NCBI
LSA_13330	Pseudogenes	2.4486	1	rRNA ribosomal RNA
LSA_1p00050	Pseudogenes	0.6482	- 1	pseudo

A 18: Differentially expressed genes expressed as log2 fold changes during incubation with fructose of *L. sanfranciscensis* TMW 1.1304 ordered alphabetically by COG functional categories. Anaerobic conditions were used as reference condition and set as 1. Up- regulated genes are shaded in grey, downregulated genes are left blank. Genes which were also differentially expressed during aerobic incubation are marked bold.

LSA number	COG functional category	1/100 Candida log2 fold changes	1/10 Candida log2 fold changes	Up/ down	gene name, results of BLAST search
LSA_00240	Hypothetical proteins	0.5984	2.0029	0	major facilitator superfamily permease. peptide efflux protein (BLAST)
LSA_00360	Hypothetical proteins	0.8986	0.6177	- 1	Permease of the major facilitator superfamily BLAST
LSA_00510	Hypothetical proteins	1.6675	1.3863	0	glucosyltransferase (Strepmut). serine endopeptidase BLAST
LSA_00540	Hypothetical proteins	1.5267	1.2075	0	RNA- binding protein. ASCH domain. hypothetical protein BLAST
LSA_00720	Hypothetical proteins	1.3914	1.8965	1	mRNA interferase. cell growth regulatory protein (BLAST)
LSA_00740	Hypothetical proteins	0.5903	0.4822	- 1	47 aa. hypothetical protein BLAST
LSA_01030	Hypothetical proteins	1.5197	0.8746	0	168 aa. abortive infection protein. immunity protein BLAST
LSA_01040	Hypothetical proteins	0.8539	2.1651	0	121 aa. hypothetical protein BLAST
LSA_01100	Hypothetical proteins	0.9831	0.3004	- 1	HxlR transcriptional regulator BLAST
LSA_01110	Hypothetical proteins	1.9459	1.8546	0	transcriptional regulator (HTH. HrX. MarR) (BLAST)
LSA_01300	Hypothetical proteins	1.164	3.3341	1	84 aa. oxidoreductase (NADP dependent) iolS. inositol utilization protein (BLAST)
LSA_01320	Hypothetical proteins	1.2427	2.1505	1	136 aa. ribokinase. hypoth pr. transcr regulator BLAST

LSA number	COG functional category	1/100 Candida log2 fold changes	1/10 Candida log2 fold changes	Up/ down	gene name, results of BLAST search
LSA_01420	Hypothetical proteins	0.9254	1.8488	0	hypothetical protein BLAST
LSA_01570	Hypothetical proteins	0.6939	0.587	- 1	integrase/recombinase plasmid associated. DNA recombinase BLAST
LSA_01980	Hypothetical proteins	1.2807	1.664	1	uvrA; excinuclease ABC subunit A. ABC transporter protein BLAST
LSA_02330	Hypothetical proteins	1.2571	1.7315	1	GntR family transcriptional regulator. aromatic amino acid aminotransferase. aminotransferase BLAST
LSA_02430	Hypothetical proteins	1.0591	2.1175	1	HAD superfamily hydrolase. cof family hydrolase BLAST
LSA_02440	Hypothetical proteins	3.3725	9.3172	1	42 aa. hypothetical protein
LSA_02660	Hypothetical proteins	0.7627	0.6001	- 1	sugar phosphate isomerase. glutamate synthase domain- containing
LSA_02710	Hypothetical proteins	1.7497	1.3813	0	83 aa. hypothetical protein BLAST
LSA_02780	Hypothetical proteins	1.7211	7.2866	1	oxidoreductase (BLAST)
LSA_02800	Hypothetical proteins	0.6929	0.4074	- 1	47 aa. hypothetical protein BLAST
LSA_02880	Hypothetical proteins	0.6475	0.3574	- 1	DegV family protein
LSA_02890	Hypothetical proteins	1.1774	0.4332	0	50 aa. hypothetical protein
LSA_02900	Hypothetical proteins	0.9948	2.117	0	150 aa. major facility superfamily protein. multidrug efflux transporter BLAST
LSA_02990	Hypothetical proteins	1.5178	1.4827	0	80 aa. hypothetical protein
LSA_03040	Hypothetical proteins	1.1502	1.7972	1	123 aa. hypothetical protein
LSA_03080	Hypothetical proteins	1.1669	2.0374	1	universal stress protein UspA. ribose- phosphate isomerase. nucleotide binding protein BLAST
LSA_03090	Hypothetical proteins	1.0171	1.5348	1	177 aa. phospholipid- binding protein. PEBP family protein BLAST
LSA_03320	Hypothetical proteins	0.7073	0.5317	- 1	142 aa. transcriptional regulator CopR. Y BLAST
LSA_03340	Hypothetical proteins	0.6248	0.5276	- 1	41 aa. hypothetical protein BLAST
LSA_03460	Hypothetical proteins	1.0123	2.8726	1	179 aa. RNA polymerase (sigma SU) BLAST
LSA_03470	Hypothetical proteins	1.3595	1.9661	1	peptidyl- prolyl- cis- trans- isomerase (BLAST)
LSA_03480	Hypothetical proteins	0.7397	0.3807	- 1	77 aa. hypothetical protein
LSA_03500	Hypothetical proteins	0.5844	1.4032	0	peptidyl- prolyl- cis- trans- isomerase (BLAST)
LSA_03510	Hypothetical proteins	0.0505	1.7619	0	67 aa. peptidyl- prolyl cis- trans isomerase BLAST
LSA_04640	Hypothetical proteins	1.226	0.442	0	65 aa. hypothetical protein BLAST
LSA_05430	Hypothetical proteins	1.0975	1.7822	1	integral membrane protein. membrane protein BLAST
LSA_05560	Hypothetical proteins	1.4716	1.8991	1	146 aa. chorismate mutase BLAST
LSA_05600	Hypothetical proteins	0.7349	0.6307	- 1	115 aa. membrane protein. hypothetical protein BLAST
LSA_05630	Hypothetical proteins	0.8109	0.61	- 1	198 aa. hypothetical protein BLAST
LSA_05680	Hypothetical proteins	0.5951	0.571	- 1	hypothetical protein. BLAST
LSA_05750	Hypothetical proteins	1.6888	2.2981	1	87 aa. hypothetical protein
LSA_05830	Hypothetical proteins	0.472	0.6233	0	40 aa. hypothetical protein BLAST

LSA number	COG functional category	1/100 Candida log2 fold changes	1/10 Candida log2 fold changes	Up/ down	gene name, results of BLAST search
LSA_05960	Hypothetical proteins	0.581	0.3235	- 1	110 aa. membrane. hypothetical protein BLAST
LSA_06450	Hypothetical proteins	0.753	0.6384	- 1	75 aa. hypothetical protein BLAST
LSA_06480	Hypothetical proteins	1.1203	1.931	1	58 aa. hypothetical protein BLAST
LSA_06500	Hypothetical proteins	1.2061	3.0231	1	2- deoxyuridine 5- triphosphate nucleotidohydrolase. dUTPase BLAST
LSA_07300	Hypothetical proteins	0.7257	0.5528	- 1	155 aa. major facilitator superfamily permease. hypothetical protein BLAST
LSA_07980	Hypothetical proteins	1.1058	2.6473	1	Transcriptional regulator BLAST
LSA_08040	Hypothetical proteins	14.2969	0.8954	0	42 aa. hypothetical protein BLAST
LSA_08100	Hypothetical proteins	0.4362	0.6923	0	104 aa. hypothetical protein BLAST
LSA_08610	Hypothetical proteins	1.056	1.8752	1	186 aa. hypothetical protein BLAST
LSA_08640	Hypothetical proteins	0.4274	0.7295	0	CRISPR- associated protein BLAST
LSA_08660	Hypothetical proteins	0.5214	1.2875	0	CRISPR assoc protein BLAST
LSA_08810	Hypothetical proteins	0.6032	0.6253	0	144 aa. competence protein ComGF BLAST
LSA_08820	Hypothetical proteins	0.7291	0.4113	- 1	56 aa. hypothetical protein BLAST
LSA_08830	Hypothetical proteins	1.4159	2.5885	1	competence protein ComGD BLAST
LSA_08850	Hypothetical proteins	0.1961	0.2313	0	competence protein ComGB BLAST
LSA_08860	Hypothetical proteins	0.9424	0.4132	- 1	competence protein ComGA BLAST
LSA_09620	Hypothetical proteins	1.0938	1.6619	1	58 aa. hypothetical protein BLAST
LSA_09750	Hypothetical proteins	1.1606	1.5251	1	108 aa. hypothetical protein BLAST
LSA_09770	Hypothetical proteins	1.1516	1.5534	1	104 aa. hypothetical protein BLAST
LSA_09890	Hypothetical proteins	0.6033	0.6546	0	153 aa. hypothetical protein BLAST
LSA_10270	Hypothetical proteins	1.0338	1.5365	1	189 aa. dithiol- disulfide isomerase BLAST
LSA_10320	Hypothetical proteins	0.8539	2.235	0	monooxygenase. hypoth prot BLAST
LSA_10400	Hypothetical proteins	0.7656	0.6556	- 1	64 aa. hypothetical protein BLAST
LSA_10650	Hypothetical proteins	0.882	1.711	0	97 aa. hypothetical protein BLAST
LSA_10740	Hypothetical proteins	1.0627	1.5623	1	123 aa. putative membrane protein. integral membrane protein BLAST
LSA_11380	Hypothetical proteins	0.5534	1.5581	0	leader peptidase (prepilin peptidase) / N-methyltransferase BLAST
LSA_11400	Hypothetical proteins	0.5921	1.6034	0	81 aa. hypothetical protein. membrane protein BLAST
LSA_11580	Hypothetical proteins	0.6138	1.0624	0	hypothetical protein BLAST
LSA_11880	Hypothetical proteins	0.8008	0.3901	- 1	179 aa. transcriptional regulator (TetR) BLAST
LSA_12230	Hypothetical proteins	1.134	3.8597	1	72 aa. hypothetical protein BLAST
LSA_12400	Hypothetical proteins	1.1593	1.7107	1	173 aa. hypothetical protein BLAST
LSA_12460	Hypothetical proteins	1.5356	0.9377	0	599 aa. hypothetical protein BLAST
LSA_12470	Hypothetical	1.5769	1.9005	1	74 aa. hypothetical protein BLAST

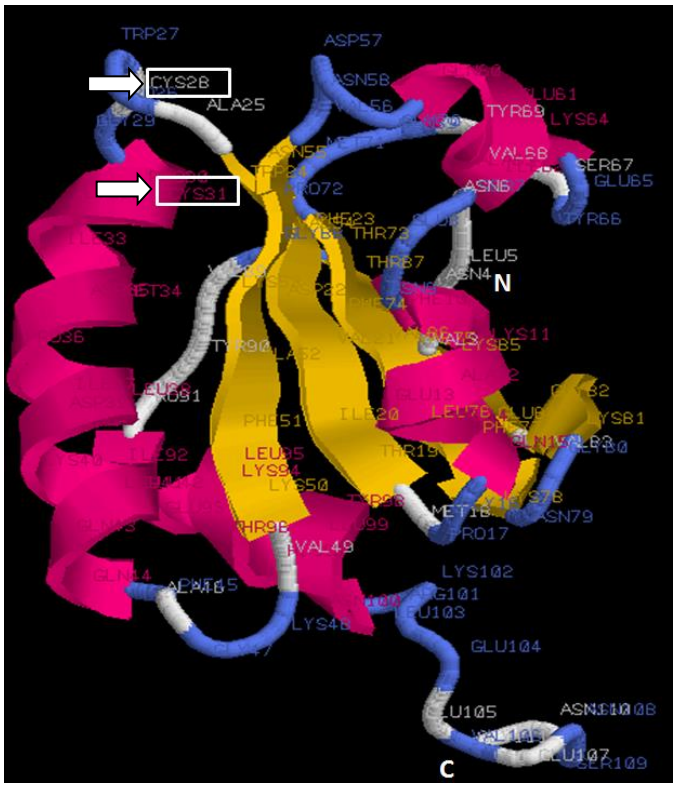
LSA number	COG functional category	1/100 Candida log2 fold changes	1/10 Candida log2 fold changes	Up/ down	gene name, results of BLAST search
	proteins				
LSA_12690	Hypothetical proteins	0.5161	1.0364	0	60 aa. hypothetical protein BLAST
LSA_13130	Hypothetical proteins	0.8883	0.586	- 1	nucleobase:cation symporter- 2. NCS2 family; xanthine/uracil permease BLAST
LSA_13170	Hypothetical proteins	0.792	0.5702	- 1	147 aa. response regulator. DNA binding BLAST
LSA_2p00160	Hypothetical proteins	0.7774	0.4156	- 1	41 aa. hypothetical protein BLAST
LSA_2p00560	Hypothetical proteins	1.116	1.533	1	189 aa. hypothetical protein. BLAST
LSA_2p00630	Hypothetical proteins	1.0089	0.9235	0	Cobyrinic acid ac- diamide synthase. replication-associated protein BLAST
LSA_01310	Pseudogenes	1.7545	2.8218	1	aldo/ ketoreductase family enzyme (NCBI BLASTN)
LSA_02120	Pseudogenes	0.7459	0.5272	- 1	pseudo
LSA_02570	Pseudogenes	1.2707	0.6272	0	pseudo
LSA_02670	Pseudogenes	1.2951	0.6421	0	pseudo
LSA_03290	Pseudogenes	0.6927	0.6265	- 1	pseudo
LSA_03300	Pseudogenes	0.6464	0.8445	0	pseudo
LSA_04960	Pseudogenes	0.7967	0.5031	- 1	pseudo
LSA_04970	Pseudogenes	2.27	1.5668	0	pseudo
LSA_05000	Pseudogenes	0.9922	1.7972	0	pseudo
LSA_05040	Pseudogenes	1.5598	1.3863	0	pseudo
LSA_05050	Pseudogenes	1.5297	0.9648	0	pseudo
LSA_05220	Pseudogenes	2.0158	2.3342	1	no information NCBI. BLAST
LSA_05590	Pseudogenes	1.5519	1.2455	0	pseudo
LSA_05690	Pseudogenes	0.1609	1.0955	0	pseudo
LSA_05740	Pseudogenes	1.1641	2.2315	1	hypothetical protein NCBI BLAST
LSA_06870	Pseudogenes	0.8808	0.5296	- 1	pseudo
LSA_06930	Pseudogenes	1.002	1.6272	1	pseudo
LSA_07330	Pseudogenes	0.7172	1.5666	0	pseudo
LSA_08000	Pseudogenes	1.5097	1.1091	0	pseudo
LSA_08600	Pseudogenes	1.1319	1.5638	1	pseudo
LSA_08770	Pseudogenes	0.1784	3.247	0	acetate kinase uncharact transporter. transposase NCBI
LSA_08780	Pseudogenes	0.6579	1.1059	0	pseudo
LSA_09780	Pseudogenes	0.9573	1.5164	0	pseudo
LSA_11710	Pseudogenes	0.4386	0.3411	- 1	pseudo
LSA_12370	Pseudogenes	0.9563	1.7109	0	pseudo
LSA_12520	Pseudogenes	1.2933	7.6293	1	62 bp of lactate dehydrogenase NCBI
LSA_13120	Pseudogenes	0.8922	0.562	- 1	pseudo
LSA_13210	Pseudogenes	1.041	0.2254	0	pseudo
LSA_13230	Pseudogenes	1.2168	2.3143	1	UPF0324 membrane protein NCBI
LSA_2p00050	Pseudogenes	0.8546	0.6062	- 1	pseudo
LSA_2p00490	Pseudogenes	1.0098	1.9121	1	pseudo

A 19: Differentially expressed genes expressed as log2 fold changes during incubation with *Candida humilis* (1/ 10 and 1/ 100) of *L. sanfranciscensis* TMW 1.1304 ordered alphabetically by COG functional categories. Anaerobic conditions were used as reference condition and set as 1. Up- regulated genes are shaded in grey, downregulated genes are left blank.

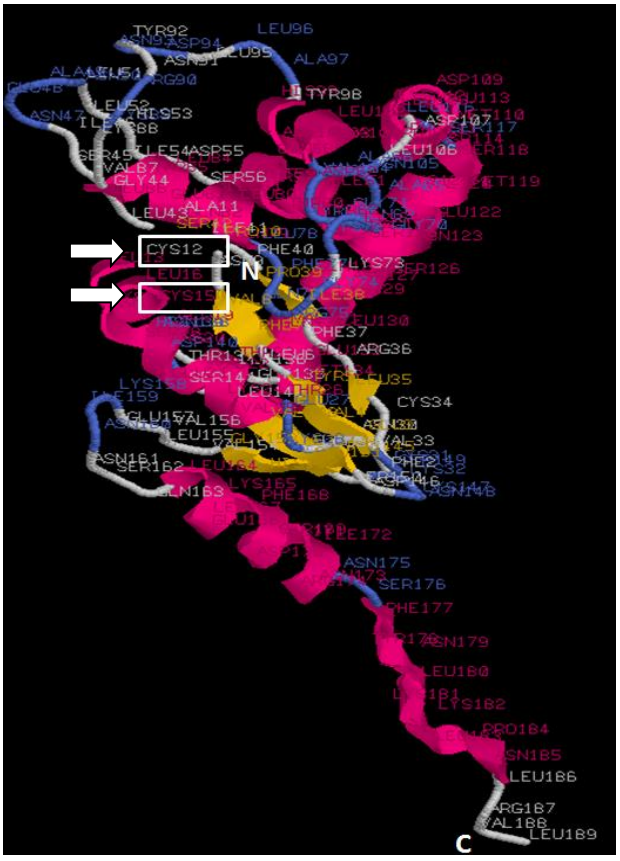
gene	Δ*tcyB* + FPKM 1	Δ*tcyB* - FPKM 2	log2 (FPKM 2 / FPKM1)	Gene description
oppD,oppF	**9766.52**	**2052.33**	**- 2.2505**	**oligopeptide transport ATP- binding protein oppD**
oppB	4851.86	1286.68	- 1.9148	oligopeptide transport system permease oppB
oppC	4578.56	1107.24	- 2.0479	oligopeptide transport system permease oppC
oppA	7080.01	2798.42	- 1.3391	oligopeptide- binding protein oppA
pepN	4406.86	1977.21	- 1.1562	aminopeptidase
LSA_00850	10815	1481.55	- 2.8678	hypothetical protein
adh2	2835.89	6574.28	1.2130	aldehyde- alcohol dehydrogenase 2
LSA_02330	266.01	42.1457	- 2.6580	hypothetical protein
LSA_02710	248.502	69.8418	- 1.8310	hypothetical protein
LSA_03220	1134.22	574.819	- 0.9805	hypothetical protein
LSA_03680	431.888	821.115	0.9269	hypothetical protein
nupG	685.591	1134.18	0.7262	Purine nucleoside transport protein nupG
purR	373.161	752.727	1.0123	Pur operon repressor
glmU	695.932	1499.17	1.1071	Bifunctional protein glmU
LSA_04670	557.947	185.147	- 1.5914	L- 2- hydroxyisocaproate dehydrogenase
LSA_05000	2583.78	723.188	- 1.8370	pseudogene
pta,ung,ydiB	**730.017**	**1596.79**	**1.1291**	**uracil- DNA glycosylase**
glmS	3272.26	1062.09	- 1.6233	glucosamine- - fructose- 6- phosphate aminotransferase
LSA_06830	5106.91	9053.6	0.8260	hypothetical protein
LSA_07110	239.53	99.371	- 1.2693	hypothetical protein
rpsT	5066.48	8464.83	0.7404	30S ribosomal protein S20
clpE	1555.83	609.537	- 1.3519	ATP- dependent Clp protease ATP- binding subunit clpE
LSA_10810	276.355	140.476	- 0.9762	pseudogene
pepX	894.959	400.234	- 1.1609	Xaa- Pro dipeptidase
pepT	483.674	278.593	- 0.7958	peptidase T
LSA_13190	386.042	970.825	1.3304	hypothetical protein
LSA_00240	247.704	30.0654	- 3.0424	hypothetical protein
bmr	746.498	438.916	- 0.7661	multidrug resistance protein 1
LSA_00590	706.929	93.1365	- 2.9241	hypothetical protein
zwf	1781.04	3030.83	0.7669	glucose- 6- phosphate 1- dehydrogenase
LSA_03080	1239.06	704.854	- 0.8138	hypothetical protein
yxkA	2665.42	1320	- 1.0138	hypothetical protein
LSA_04150	535.922	231.707	- 1.2097	hypothetical protein
clpP	10725	6143.65	- 0.803806	ATP- dependent Clp protease proteolytic subunit
msrB	1726.35	989.532	- 0.802903	peptide methionine sulfoxide reductase
LSA_07480	351.046	113.982	- 1.6228	hypothetical protein
hup	9498.21	17155.4	0.8529	DNA- binding protein HU
rpsU	18699.9	33242	0.8299	30S ribosomal protein S21
dtd	**2180.98**	**1136.46**	**- 0.9404**	**hypothetical protein**
relA	541.004	277.122	- 0.9651	GTP pyrophosphokinase
LSA_08440	218.973	73.077	- 1.5832	hypothetical protein
LSA_08450	230.169	673.152	- 1.7736	hypothetical protein
LSA_08570	621.264	383.855	- 0.6946	hypothetical protei
patA	456.451	123.192	- 1.8895	aminotransferase A
mnmA	5036.63	2270.57	- 1.1494	tRNA- specific 2- thiouridylase mnmA
pepB	**1139.56**	**579.562**	**- 0.975443**	**Group B oligopeptidase pepB**
clpC	1433.57	880.38	- 0.703417	Negative regulator of genetic competence clpC/mecB

gene	Δ*tcyB* + FPKM 1	Δ*tcyB* - FPKM 2	log2 (FPKM 2 / FPKM1)	Gene description
ctsR	4114.49	2083.87	- 0.981448	transcriptional regulator ctsR
LSA_13130	549.028	1105.05	1.0092	hypothetical protein
pepE	6110.74	3203.13	- 0.9318	aminopeptidase

A 20: Significantly differentially expressed genes for Δ*tcyB* treated (+) vs. Δ*tcyB* untreated (-). Depicted are the FPKM (Fragments Per Kilobase of transcript per Million mapped reads) values, log2 fold changes and gene descriptions; Isoforms of the genes *oppD,oppF*; *pta,ung,ydiB*; *dtd* and *pepB* are marked in bold.



A 21: 3D structure of LSA_02610, modelled by 3D JIGSAW (Bates et al. 2002). The protein shows the characteristic thioredoxin-fold with a three-layer α-β-α sandwich, with two parallel plus two anti-parallel β-strands. The two cysteines of the N- terminal CXXC motif (white arrows), the N- and C- terminus of the protein are marked.



A 22: 3D structure of LSA_10270, modelled by 3D JIGSAW (Bates et al. 2002). The protein shows the characteristic thioredoxin-fold with a three-layer α-β-α sandwich, with two parallel plus two anti-parallel β-strands. The two cysteines of the CXXC motif (white arrows), the N- and C- terminus of the protein are marked.

10 LIST OF PUBLICATIONS DERIVED FROM THIS WORK

Peer-reviewed journals

Stetina M., Behr J., Vogel RF. (2014). The transcriptional response of Lactobacillus sanfranciscensis DSM 20451T and its tcyB mutant lacking a functional cystine transporter to diamide stress. *Appl. Environ. Microbiol.* **80**:4114–4125.

Capuani A., Stetina M., Gstattenbauer A., Behr J., Vogel RF. (2014). Multivariate analysis of buckwheat sourdough fermentations for metabolic screening of starter cultures. *Int. J. Food Microbiol.* **185**:158–66.

Poster presentations

Stetina M., Behr J., Sieuwerts S., Smid E.J., Vogel RF. "Transcriptome analysis of *L. sanfranciscensis* TMW 1.1304 in response to electron acceptors and the presence of *Candida humilis*" Poster presented at V Symposium on Sourdough- Cereal Fermentation for Future Foods 2012, Helsinki, Finland, 10-12 October 2012. Abstract published in VTT Technology 50, Helsinki: VTT Technical Research Centre of Finland. 2012: 86.

Capuani A., Stetina M., Vogel RF. "Novel approaches in gluten-free sourdough fermentations" Poster presented at Weihenstephaner Institut für Getreideforschung, Freising, Germany, 20-21 March 2014.